Conversion of Cotton Gin Waste to Bioethanol: Pretreatment, Hydrolysis and Fermentation

Dissertation submitted

in partial fulfillment of the degree of

Doctor of Philosophy

in

Biotechnology and Medical Engineering

by

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based on research carried out
under the supervision of

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October, 2016

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I, *Shitarashmi Sahu*, Roll Number *509BM604* hereby declare that this dissertation entitled *"Conversion of Cotton gin waste to Bioethanol: Pretreatment, Hydrolysis and Fermentation* " represents my original work carried out as doctoral student of NIT Rourkela and, to the best of my knowledge, it contains no material previously published or written by another person, nor any material presented for the award of any other degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the dissertation. Works of other authors cited in this dissertation have been duly acknowledged under the section "Bibliography". I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation.

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Acknowledgements

This thesis is the end of my journey in obtaining my Ph.D. This thesis has been kept on track and been seen through to completion with the support and encouragement of numerous people including my well wishers, my friends, colleagues and some institutions. At the end of my thesis, I would like to thank all those people who made this thesis possible and an unforgettable experience for me. At the end of my thesis, it is a pleasant task to express my thanks to all those who contributed in many ways to the success of this study and made it an unforgettable experience for me.

First and foremost I want to thank my advisor ***Prof. Krishna Pramanik****. I appreciate all her contributions of guidance, ideas, and funding to make my Ph.D. experience productive and stimulating. Under her expert guidance, I successfully overcame many difficulties and learned a lot. Without her, this thesis would not have been materialized. I can only say proper thanks to her through my future work.*

My special gratitude to Professor ***S. K. Sarangi****, Director, National Institute of Technology, Rourkela for all the facilities provided to successfully complete this work.*

I express my sincere thanks to Prof. ***M. K. Gupta****, Head, Department of Biotechnology & Medical Engineering and members of Doctoral Scrutiny Committee (DSC) Prof. S.K Patra, Prof. S. Das , Prof. Amit Biswas and all the faculty member of Biotechnology & Medical engineering department for their suggestions and constructive criticism during the preparation of the thesis.*

This work also would not have been possible without the help of all the research group members. I would like to express my gratitude to my research group and Bikram Nayak for his assistance in my research work. I am also thankful to my other research colleagues Bhishma Patel, Rashmi Ranjan, Amit Singh, Sakira Begam, Parinita Agrawal, Neelam Meher and Sanjeeb Kumar Bhoi for their support and good wishes.

I greatly thankful to my husband Tusar Kanta Samal, who had always been very supportive and helpful in both research work and life.

Finally, I express my humble regards to my parents, sister and in-laws for their immense support, sacrifice and their unfettered encouragement at all stages.

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Abstract

The present research focuses on the conversion of cotton gin waste, a potential lignocellulosic biomass produced in cotton industry, to bioethanol. The major technological hurdle for utilizing this waste to bioethanol is the pretreatment process to release sugar components for ethanol fermentation. Even the most effective pretreatment method using dilute sulphuric acid suffers from several drawbacks such as the process is hazardous and produces toxic by-products which affect the growth of yeast during fermentation leading to lower bioethanol yield. Therefore, an alternative pretreatment strategy is essential for the removal of lignin, thereby releasing cellulose and hemicellulose as fermentable sugar components from cotton gin waste. In this context, pretreatment of biomass using organic acid might be attractive as it produces less toxic by-products and the method is environment-friendly. It is further reported that biological pretreatment is advantageous over chemical pretreatment methods because of the requirement of mild reaction conditions, low energy and formation of minimal toxic byproducts. Therefore, in the present research, pretreatment of cotton gin waste using both biological and organic acid treatment was performed and the results were compared with the most widely used dilute sulphuric acid pretreatment.

Among the four organic acids, maleic acid pretreatment was found to be the most efficient yielding maximum pentosan sugar of 125.50±0.67 g/g (83% C5 sugar release) which was comparable to the most widely used sulfuric acid (132.08±1.06 g/g yield) pretreatment at optimum condition o130°C, 45 min and 500mM. However, the sulfuric acid pretreatment produced more toxic by-products in comparison to organic acids. The fermentation of 41.75 g/l mixed hydrolysate (C5 and C6) obtained from maleic acid pretreated biomass using sequential culture of *Saccharomyces cerevisiae* and *Pichia stipitis* yeast strains achieved maximum 18.74 g/l ethanol concentration, 0.48 g/g ethanol yield, 2.25 g/l/h ethanol productivity, 88% maximum theoretical yield and 0.30 g/g biomass yield at 30°C, 200 rpm and 5.5pH in a bench top bioreactor.

An effort was given to isolate fungi from the soil of dumping area of cotton gin waste generated in cotton mill. Among the isolated fungi, *Aspergillus flavus* (UNF1) was found to be most efficient fungal strain for the pretreatment of CGW achieving 67.04% lignin removal with the release of 66% and 74.5% of cellulose and hemicellulose at pH 4.5, 122

rpm and 35°C. Further, 34.83 g/l total sugar by enzymatic hydrolysis and 15.44 g/l ethanol concentration, 0.45 g/g yield, 1.74 g/l/h productivity, 0.35 g/g biomass yield were obtained by fermentation in the bioreactor.

Overall, it has been demonstrated that the pretreatment of cotton gin waste with maleic acid followed by delignification is comparatively more effective providing the maximum pretreatment efficiency with less time and finally bioethanol production than the fungal pretreatment method. A substantial bioethanol production was achieved by biological pretreatment using the *Aspergilus flavus* (UNF1) fungal strain isolated from the soil of the dumping area of cotton gin waste in the cotton industry as a new source. The biological pretreatment is favorable than the organic acid pretreatment from an economical point of view by avoiding an additional step of chemical delignification involved in organic acid pretreatment. Furthermore, the biological method may be a promising alternative to the widely used sulfuric acid pretreatment which requires additional delignification and detoxification steps. The higher pretreatment time required for biological pretreatment (24days) in comparison to the acid pretreatment (few hours) may be reduced by genetically modifying the isolated fungal strain thereby making the process more economically viable.

Thus, it has been concluded that the delignification process using *Aspergilus flavus* UNF1 as pretreatment agent and the microbial system involving the sequential use of *S. cerevisiae* and *P. stipitis* yeast strains for fermentation may be an attractive option for large-scale bioethanol production from cotton gin waste in future.

Keywords: *Cotton gin waste, lignocellulosic biomass, bioconversion, lignin, cellulose, hemicelluloses, white rot fungi, organic acid, pretreatment, hydrolysis, fermentation, bioethanol, response surface model, toxic by-products*

CONTENTS

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List of Abbreviations

ANOVA	Analysis of variance
BSA	Bovine serum albumin
CGW	Cotton gin waste
CGT	Cotton gin trash
CGD	Cotton gin dust
CCD	Central composite design
DNS	Di-nitro salicylic acid
FTIR	Fourier transform Infrared spectroscopy
HPLC	High pressure liquid chromatography
IMTECH	Institute of Microbial Technology
KBr	Potassium bromide
LCW	Lignocellulosic waste
NCIM	National collection of industrial microorganisms
PDA	Potato dextrose agar
RSM	Response surface methodology
SEM	Scanning electron microscope
SF	Severity factor
SSF	Simultaneous saccharification and fermentation
SMC	Submerge state cultivation
SSC	Solid state cultivation
XRD	X-ray diffractions

Chapter 1

Introduction

1 General Introduction

1.1 Background and significance of study

Due to the gradual depletion of petroleum oil reserves, its rising price, uncertainty in availability and environmental consequences has drawn attention worldwide towards the production of ethanol as an alternative source of transportation fuel. This has prompted a lot of research interest in the last two decades in the development of biofuels as promising alternatives to petroleum-based fuels because these are derived from renewable resources, environmental benign and offer reduced greenhouse gas emission. However, biofuel should be economically competitive, technically feasible, environmentally adaptable and vigorously available. Therefore, in recent years efforts have been put in place to produce bioethanol, biodiesel, biohydrogen and methane from lignocellulosic biomass rather than from energy crops because of the consumption of land and water in high demand for their growth [1]. Furthermore, the use of corn and sugarcane to produce biofuel is increasingly being discouraged due to current worldwide rise in food price [2]. In order to minimize food-feed-fuel conflicts, it is necessary to integrate all kinds of bio-waste into a biomass economy [3]. Though the technology for the conversion of lignocellulosic waste has long been considered to be rather expensive, however, recent increase in grain prices leads to divert the attention towards lignocellulosic waste for the production of biofuels that will reduce competition with grain for food and feed, and allow the utilization of variety of materials which would otherwise go to waste.

Lignocellulosic waste such as agricultural waste, municipality waste, weeds, wood, grasses, agricultural residues and industrial waste is considered as potential feedstock for bioethanol production [4,5]. It has been estimated that the total bioethanol production

from lignocellulosic waste can produce 491 GL year^{-1}, which is about 16 times higher than the current scenario of bioethanol production [6]. Cotton gin waste is a lignocellulosic biomass and a huge quantity of this waste is generated worldwide (3.23 million tons) in cotton industries. Due to stringent environment regulations, the disposal of this waste is one of the biggest problems that are faced by cotton industries all over the world including India which is the second largest cotton producing country [1]. This waste can be a promising alternative source for bioethanol production [1], if an effective conversion process is developed. However, not much study has been reported to exploit this potential feedstock for the production of bioethanol so far. There are three major challenging steps involved in the conversion of any lignocellulosic waste including cotton gin waste (CGW) to bioethanol which are - (i) pretreatment for the release of cellulose and hemicelluloses components by removing lignin, (ii) hydrolysis for converting released sugar components to fermentable sugars and (iii) fermentation of sugars to bioethanol. While pretreatment of lignocellulosic waste using dilute sulfuric acid is the most efficient and widely used method [6], besides hazardous, this method produces toxic by-products [5] which affect fermentation thus resulting low bioethanol yield. In this context, pretreatment using organic acid or microbial strains may be beneficial for bioethanol production as reported [7–9]. Another important challenge lies in the development of efficient and stable microbial strains that have the ability to co-ferment pentose and hexose sugar components released by hydrolysis to bioethanol.

1.2 Bioethanol as the future transportation fuel

The current and future economic development critically depends on the long-term availability of energy sources that are affordable, accessible and environmentally friendly [2]. Bioethanol is an “oxygenated” fuel due to its higher oxygen content. The combustion of fuel gasoline offers gaseous pollutants such as carbon monoxide (CO), hydrocarbons and particulates. Therefore, the addition of bioethanol or other oxygenated fuels to gasoline can reduce CO production by providing more oxygen and promote complete combustion [10]. Bioethanol is a clear colorless liquid, flammable, biodegradable, relatively harmless to the environment. Bioethanol is a high octane and water-free alcohol which is produced from the fermentation of sugar or starch. It is suitable as a blending ingredient of gasoline or as a raw material to produce high octane fuel ether additives [11]. Bioethanol emits 35% less carbon monoxide, 79% less carbon dioxide, 42% less

nitrogen oxides, 39% less particulate matter and 43% less hydrocarbons than the petroleum oil [12]. Combustion of oxygenated fuels produces carbon dioxide (CO_2) as the end product rather CO. The benefits lie not only in the reduction of CO concentration thereby offering less health risks but also in the contribution of CO_2 to the atmosphere. Plants, trees and various other organisms assimilate atmospheric CO_2 to use as a carbon source. Utilizing the waste products from agriculture and feedstock (biomass) for bioethanol production, therefore, do not contribute a net CO_2 into the atmosphere. In view of the environmental benefits and the depletion of crude oil, industry has been moving towards potential bioethanol fuel production [10]. Therefore, cellulosic bioethanol is represented to be a promising choice from the perspectives of both net energy gain and overall emissions of contaminants [13]. Bioethanol fuel blends are effectively used in some countries and the most common blends are E5 (5% bioethanol and 95% petrol) and E85 (85% volume bioethanol and petrol) [2,13]. An advance technological and well-organized research on bioethanol are still in progress, an efficient combination of approachable systems analysis and design of economical techniques should emerge for potential second-generation (lignocellulosic biomass) biofuel production [14]. Thus, up to 491 GL $year^{-1}$ of bioethanol can be produced from lignocellulosic biomass, which is about 16 times higher than the current world bioethanol production and 32% of the global gasoline consumption can be replace using bioethanol in E85 fuel [1].

1.3 Lignocellulosic biomass

Lignocellulosic biomass constitutes the world's largest renewable resource and abundantly available biomass on the Earth . It consists of cellulose, hemicellulose (complex carbohydrates) and lignin. Any biomass containing sugars or converted to sugars, can further use as fermentation substrates for bioethanol production. 1^{st} generation bioethanol is generally produced from sugarcane in Brazil or corn in USA [4]. However, to enable a more substantial increase in worldwide bioethanol production capacity, lignocellulosic substrates need to be exploited. There are various types of lignocellulosic raw materials that are differentiated by their composition, origin and structure. Lignocellulosic feedstocks can be categorized into five main groups: energy crops, agricultural residues, forest wood (hard wood and soft wood), industrial waste and municipal waste. The main groups of raw materials for bioethanol production are recognized such as crops grown on fertile soils (sugarcane, corn, soya beans, oilseed, switchgrass, maze and hybrid poplar),

waste biomass (straws, corn stover, and waste wood), some herbaceous, municipal solid waste, weeds (*Ipomoea carnea, Eicchornia crassipes, Lantana camara, Prosopis juliflora* and *Saccharum spontaneum*) and industrial waste (sugar cane bagasses, wood residues, cotton gin waste, paper sludge) etc. These cellulosic substrates do not require additional economic input as they grow on agriculturally land or water bodies. These feedstocks can produce a substantial bioethanol, which could solve the problem of their disposal as well as environmental pollution. Generally, most of the lignonocellulosic biomass is not directly fermentable because sugar components are in polymeric form. Furthermore, lignocellulosic biomass is a carbon neutral source of energy as the combustion of lignocellulosic bioethanol produces no net carbon dioxide into the atmosphere. Fermentation of these residues to bioethanol is an attractive way to supplement the fossil fuels.

1.4 Cotton gin waste as a potential feedstock for bioethanol production

Globally, four major cotton-producing countries India, China, USA and Pakistan are considered for approximately three-quarters of world's cotton producer. India is the 2nd largest cotton producing country in the world and has a large number of cotton mills. A huge quantity of cotton gin waste is generated during the processing of the cotton. The disposal of cotton gin waste is one of the biggest problems faced by cotton industries, which causes air and environmental pollution. Cotton gin waste is a lignocellulosic biomass and thus, can be utilized to produce bioethanol as a promising alternative energy source. The waste generated after the ginning of cotton fibers can be potentially utilized as a feedstock for the production of fuel bioethanol since it is rich in cellulose [15]. The residues from cotton crop cultivation are of two types: cotton plant trash (CPT) and cotton gin trash (CGT). CPT remains as residues in the field after the harvest of cotton, whereas CGT is generated by the cotton ginning process. From these two types of wastes, CGT is very important to researchers and cotton producers due to its high production and difficulty in disposing of it [16]. Raw cotton processing generates cotton gin residue (CGR), which is composed of immature bolls, cotton seed, hulls, burs, sticks, leaves, cotton lint and dirt [17].

1.5 Composition of cotton gin waste

The composition of the biomass is one of the important factors to determine the suitability of biomass as a fermentation feedstock for bioethanol production. Higher fermentable sugars content of the biomass is most desirable for bioethanol production. Cotton gin waste consists of three major structural polymeric components namely lignin, cellulose and hemicelluloses [18,19]. The typical composition of cotton gin waste is 40-50% cellulose, 20-30% hemicelluloses and 20-30% of lignin [20,21]. In order to exploit cotton gin waste for its fermentable sugars, its chemistry must be understood. Bioethanol yield from biomass is directly related to hemicelluloses and cellulose content in the feedstock [22]. The lignin cannot be used for bioethanol production due to different composition [1].

1.5.1 Cellulose

Cellulose is an organic polymer having a highly crystallized structure as a result of the existence of hydrogen bonds as depicted in figure 1.1. In distinction to its amorphous region, the crystalline region of cellulose makes it difficult to hydrolyze [23]. Hydrogen bonds between different layers of the polysaccharides contribute to the resistance of crystalline cellulose to degradation. Cellulose (beta (1-4)-linked chain of glucose molecules) is a polymer of D-glucose units linked by 3-glucoside bonds from the anomeric carbon of one unit to the C-4 hydroxy of the next unit [24]. The cellulose chains further aggregate into alternating highly crystalline and amorphous regions in a manner described by the fringed micelle theory [24]. The cellulose fibers are sometimes referred to as the elementary fibrils and/or microfibrils [25]. In the biomass feedstock, cellulose is the main reservoir of glucose, which is the most desired fermentation component [10]. Cotton gin waste composed of typically 40-50% cellulose, 20-30% hemicelluloses and 20-30% of lignin [20,21].

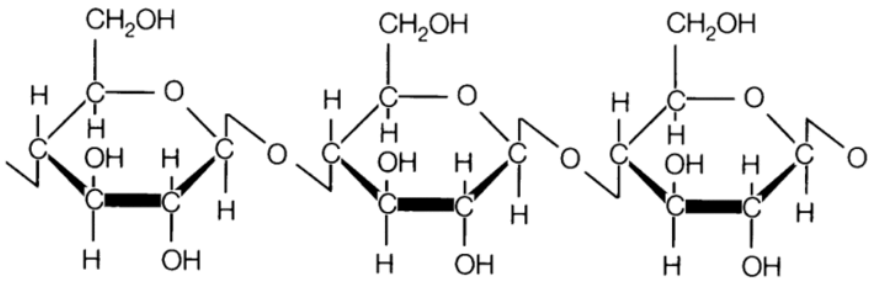

Figure 1.1: The cellulose is an organic polymer having a highly crystallized structure

1.5.2 Hemicellulose

Hemicelluloses are the most complex and highly branched polysaccharides that occur in association with cellulose in the cell walls (figure 1.2) [26]. The monomers that comprise of hemicellulose are hexoses (glucose, galactose and mannose) and pentoses (arabinose and xylose). Hemicellulose can be classified into three groups namely, xylans, mannans and galactans based on the polymer backbone that is very often homopolymeric with β-1,4 linkages. In softwoods, the primary hemicellulose components are galactoglucomannans and arabinoglucuronoxylan, while the principal hemicelluloses in hardwoods are glucomannans and methyl glucornoxylans [27]. Xylan is important in terms of the percentage of total hemicellulose found in biomass waste. In the cell wall, the hemicellulose polymers surround and associate with the cellulose core of the microfibrils by means of hydrogen bonds [28].

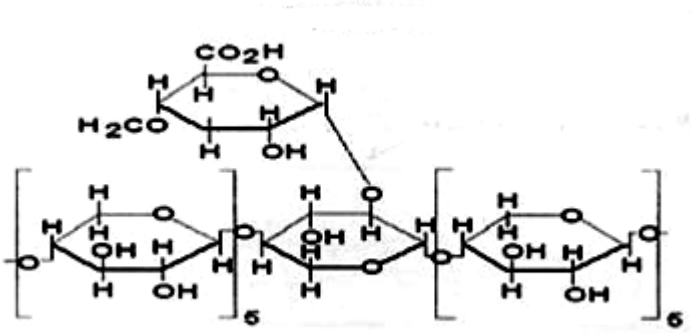

Figure 1.1: Structure of most complex and highly branched polysaccharides- hemicellulose

1.5.2 Lignin

Lignin serves as the bonding element or "cement," between plant fibers and act as a barrier to degradation of the cell walls [29]. Lignin provides structural rigidity to plant cell wall by forming firm linkages with cellulose and hemicelluloses as depicted in figure 1.3 [9]. Lignin is an aromatic and rigid three-dimensional phenyl propane bipolymer with phenyl propane units held together by ether and carbon-carbon bonds [30]. It is constructed of three monomers: coniferyl alcohol, sinapyl alcohol and coumaryl alcohol each of which has an aromatic ring with different substituent [31]. The dominant monomeric units in the polymers are benzene rings bearing methoxyl, hydroxyl and propyl groups that can be attached to other units [32]. Lignin strengthens the cell structures by stiffening and holding the fibers of polysaccharides together [33]. The complex structure of lignin is counter attacked by most microorganisms (aerobic and anaerobic) and it is not fermentable or digestible.

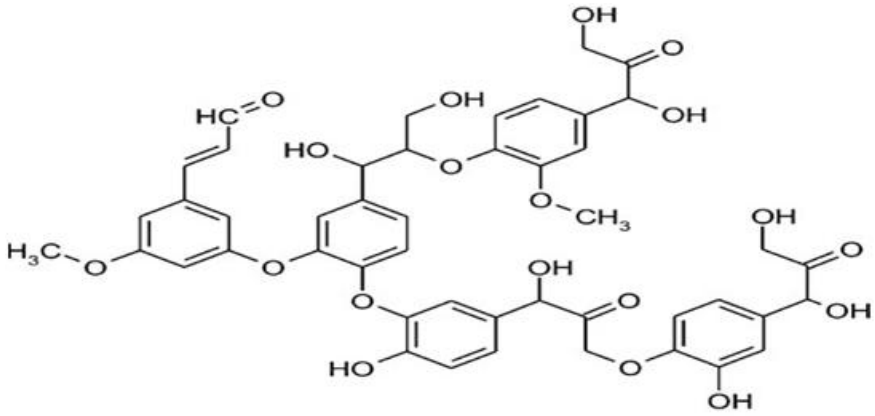

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Figure 1.2: Structure of an aromatic and rigid biopolymer - lignin

1.6 Biomass conversion techniques: pretreatment, hydrolysis and fermentation

In general, the production of bio-ethanol from cotton gin waste, like any other lignocellulosic waste, is based on three principal steps such as pretreatment, saccharification and fermentation [34]. The first step aims to reduce the quantity of lignin present in the biomass thereby makes the cellulose and hemicellulose readily available for the saccharification process. In order to produce sugars from the biomass, the waste is pre-treated with acids or enzymes. The cellulose and hemicellulose portions are broken down by enzymes or dilute acid into sugar monomers which are then fermented into bioethanol. The main factors governing the lignocelluloses breakdown to fermentable

monosaccharides are the reduction in cellulose crystallinity and the removal of lignin [35]. The second step is to extract the monosaccharides present in the cellulose (glucose) and the hemicellulose (xylose, arabinose, galactose and mannose) by acid or enzymatic hydrolysis. Enzymatic hydrolysis is advantageous over acid hydrolysis as it offers higher yields, minimal by-product formation, mild operating conditions and low energy requirements. The cellulase enzymes employed for the hydrolysis of cellulose to glucose are mainly categorized into three groups: endo-glucanases, exoglucanases, and beta-glucosidases. The three step process can be modified to improve the yield of bioethanol from cotton gin waste [16,36]. Once the carbohydrate polymers are hydrolyzed into free sugar monomers they can be fermented to bioethanol using various ethanologenic microorganisms. Yeast is the most commonly used organism for bioethanol fermentation, however, few species of bacteria like *Zymomonas mobilis* and *E. coli* are also used.

1.7 Response surface model

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building for optimization study [37]. A response surface model is a set of advanced design of experiments (DOE) techniques that helpful for better understanding and optimizing the process with a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. Originally, RSM was developed to model experimental responses and then migrated into the modeling of numerical experiments. The main application of RSM to design optimization is aimed at reducing the cost of expensive analysis methods with low time-consuming experiments. There are mainly two types of response surface designs exist: central composite designs and Box-Behnken designs.

1.8 Organization of thesis

The thesis has been organized into the following six chapters-

Chapter 1: Presents a brief introduction emphasizing on the lignocellulosic biomass including cotton gin waste as a potential feedstock to bioethanol, its major conversion processes including pretreatment, hydrolysis and fermentation, bioethanol production and optimization(RSM).

Chapter 2: Presents an extensive literature survey emphasizing on the research on lignocellulosic biomass, ethanol from cotton gin waste and different conversion techniques.

Chapter 3: Presents scope and objective of the study.

Chapter 4 : Describes the materials and detail experimental procedure to carry out the various stages of research work including : composition analysis of cotton gin waste I) Bioethanol production from cotton gin waste: effect of organic acid pretreatment II) Bioethanol production from cotton gin waste: effect of fungal pretreatment III) Bioethanol production from cotton gin waste: Effect of mixed culture IV) Bioethanol production from cotton gin waste: Effect of fungal strain isolated from the soil of cotton industry.

Chapter 5: Presents the "Results and Discussion" on the experimental results which has been divided into four parts that include: 5.1: Bioethanol production from cotton gin waste: effect of organic acid pretreatment, 5.2: Bioethanol production of cotton gin waste: effect of fungal pretreatment, 5.3: Bioethanol production from cotton gin waste: Effect of mixed fungal culture, 5.4: Bioethanol production from cotton gin waste: Effect of fungal strain isolated from the soil of cotton mill.

Chapter 6: Includes a brief summary and conclusion of the thesis work along with suggested future study.

Chapter 2

Literature Review

2 Literature Review

2.1 Cotton gin waste

Cotton gin waste contains about 40-50% or more holocellulose content [38,39] which makes it a potential feedstock for bio-ethanol production for the transportation sector. Different conversion processes of cotton gin waste to biofuel have been investigated by researchers like Shen and Agblevor reported the production of 157 liters of bioethanol produced per ton of cotton gin waste [40]. Worldwide production of this waste is approximately 3.23 million tons per year [38]. Whereas, 218 kg of cotton fiber generates 68-91 kg of CGT [41] and ginning one bale (227 kg) of spindle harvested seed cotton lint contributes between 37 and 147 kg of waste [42]. With this large quantity of wastes, the final disposal becomes a major problem to the cotton industry which becomes more critical during winter and rainy seasons when insects use these residues as survival sites [43,44]. Availability is one of the most important factors infeasibility of using any product for bioenergy production [45]. In this context, though the abundance of cotton gin waste throughout the world is a major problem of disposal, it is, however, a simultaneous advantageous for bio-energy production. These cotton wastes, containing minute fibers when been suspended in air may cause serious manifestations in the human body mainly affecting lungs [46]. The traditional disposal methods including land application, landfilling and incineration of the cotton gin waste have several disadvantages such as environmental pollution, health hazardous and limitation of land supply etc [40,47]. The current method of the choice is the incorporation of cotton gin waste into soil. The need for alternative disposal technologies is very pronounced in the cotton industry because of the climatic conditions and small ginning plants [48]. The high ash content of the

feedstock generates a slagging problem associated particularly with large-scale incineration. Landfilling is not a viable option because tipping fees cost are very high. On the other hand emission of greenhouse gasses is increasing rapidly with fast depletion of oil resources. Whereas, alternative fuels produced from renewable resources, such as bioethanol, provide numerous benefits in terms of environmental protection, economic development and national energy security [49]. In this context conversion of this cotton gin waste to bioethanol could be a potential source for bioethanol production [20].

The higher level of cotton production is directly related to the higher production of biomass wastes and residues. Worldwide, approximately 3.23 million tons of cotton gin waste was produced per year [16]. India has the largest area under cotton production and China is the largest producer of cotton worldwide, whereas India is the second largest cotton producer [17].

2.2 Conversion of lignocellulosic biomass to bioethanol

The conversion of lignonocellulosic biomass to bioethanol is mainly divided into three major steps such as: pretreatment, hydrolysis and fermentation. Pretreatment is a process that is used for removing or modifying lignin, extraction of hemicellulose, decrystallizing cellulose, removing actyle group from hemicellulose, reduce polymerization of cellulose, expanding the structure to increase pore value and internal surface area so that hydrolysis of carbohydrate fraction to monomeric sugars can be achieved more rapidly with higher yields [50]. It is reported that a different pretreatment method affects biomass in different ways [51,52]. Pretreatment is therefore, essential to disrupt or remove lignin from lignocellulosic biomass and thus, increase the accessibility of cellulose [53,54]. But many pretreatment processes are highly expensive and complex. Moreover, some of the delignification methods [55] are found to have an influence on the compatibility of the conversion process. If the pretreatment is not efficient enough then the resultant residue is not easily hydrolyzable by cellulase enzyme and if it is more severe, it produces toxic by-products that inhibit the growth of fermentative microbial strains and thus lower bioethanol yield [56]. The goal of the pretreatment of lignocellulosic biomass is shown in adapted figure 2.1 [57], where the lignin was removed by releasing cellulose and hemicellulose of biomass.

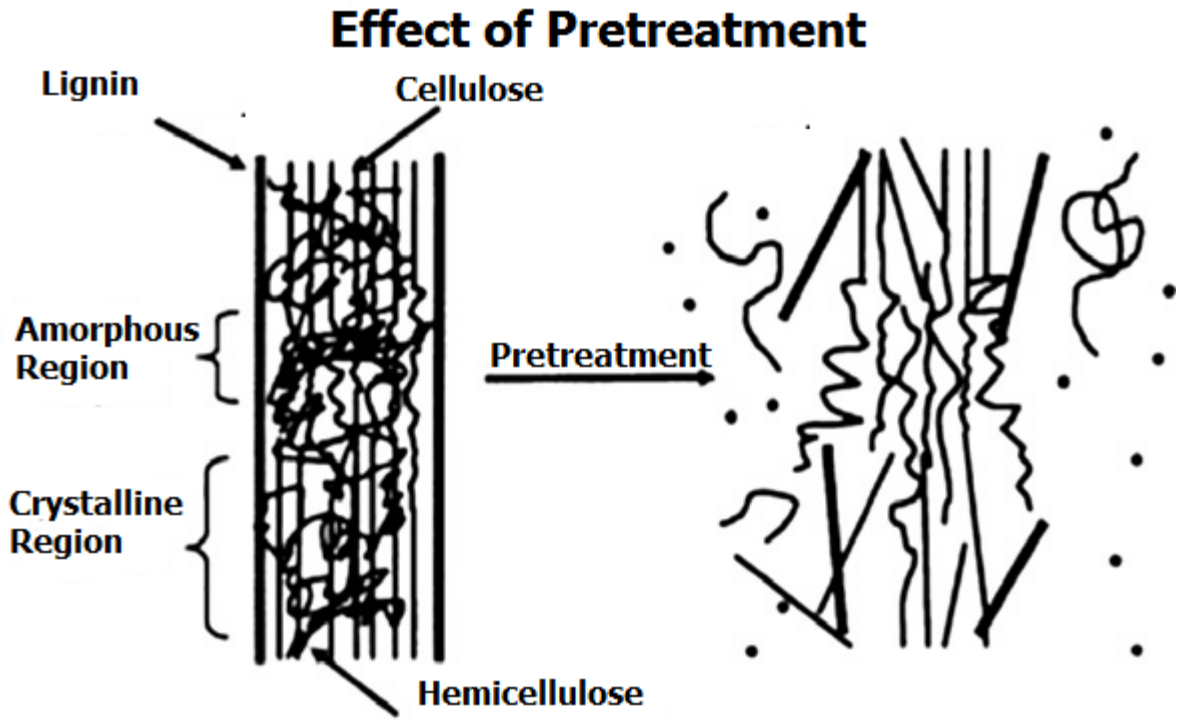


Figure 2.1: Schematic goals of pretreatment for lignocellulosic material

Several methods have been introduced for the pretreatment of lignocellulosic materials to achieve an efficient accessibility of the cellulosic components for enzymatic hydrolysis. These methods are mainly classified into physico-chemical, physical, chemical and biological pretreatment [58]. In this section, we review these methods, although not all of them have yet been developed enough to be applied for the applications in large-scale [59].

2.2.1. Physical pretreatment

Physical pretreatment of lignocellulosic waste offers the accessible surface area and size of pores, reduce the crystallinity and degrees of polymerization of cellulose [59]. Mechanical treatment reduces biomass size below 20 sieves [60] to increase the digestibility of cellulose and hemicellulose present in biomass. Toxic inhibitors (furfural and phenolic compounds) generated by pretreatment process are harmful to cells. Physical treatment can reduce the production of inhibitor through "fractional conversion" [61]. The extrusion process is one of the promising physical pretreatment methods for biomass conversion to bioethanol production. In extrusion, the materials are subjected to mixing, heating and shearing which lead to chemical and physical modifications during the passage through the extruder [36]. The treatment like screw speed and barrel temperature are effective to disrupt the lignocellulose biomass structure causing

defibrillation, fibrillation and shortening of the fibers thereby increase the accessibility of carbohydrates to enzymatic attack [22]. Irradiation with gamma rays, microwaves and electron beam can improve saccharification and delignification of lignocelluloses. The combination of radiation and acid treatment can further accelerate enzymatic hydrolysis [59]. The liquid hot water method used to hydrolyse the hemicellulose to recover a high percentage of xylose (88-98%) and the method is environmentally attractive and economically interesting [16]. Pyrolysis is also used for the pretreatment of lignocellulosic materials for the conversion of cellulose and hemicellulose into fermentable sugars with higher yields [62]. Hydrothermolysis is also one of the conventional approaches, which started as a pretreatment method before hydrolysis [63]**.** In the hydrothermal process, water, steam and heat are used [64]. But most of these methods are expensive, time-consuming and energy-intensive.

2.2.2 Physico-chemical pretreatment

The combination of physical and chemical treatments is most efficient to recover hemicellulose and alters lignin structure thereby provides an improved accessibility of cellulose for hydrolysis [65]. Steam explosion is one of the most promising physico-chemical methods to make biomass more accessible for hydrolysis [66]. Basically, in this method, the material is heated using high-pressure steam for a specific time [67]. Pretreatment using steam explosion increases the crystallinity of cellulose by promoting crystallization of the amorphous portions thereby eases hydrolysis of hemicellulose and also promotes delignification [1]. This technique is economically attractive, requires less hazardous chemicals and has higher sugar recovery [62]. The extraction of cellulose from cotton gin waste was studied using a steam explosion technology as a pretreatment process followed by alkali bleaching which produced a higher yield of bioethanol [68]. By adding H_2SO_4 (or SO_2) or CO_2 in a steam explosion of lignocellulosic waste can efficiently improve enzymatic hydrolysis, reduce the formation of toxic by-products and leads to a complete liquefaction of glucan, xylan, mannan, galactan and arabinan [30,38]. Ammonia fiber explosion (AFEX) is one of the alkaline physicochemical pretreatment processe in which the material is subjected to liquid ammonia at high pressure, temperature and a subsequently fast decompression. AFEX process is more efficient for the biomass which has less lignin and the method does not significantly solubilize hemicellulose in comparison to other pretreatment processes such as dilute-acid pretreatment. In CO_2 explosion, the release of 75% theoretical glucose during 24h of the

enzymatic hydrolysis has been reported [30]. Maximum 83% theoretical bioethanol yield has been achieved by physico-chemical treatment from lignocellulosic waste [38].

2.2.3 Chemical pretreatment

The most common chemical pretreatment method involves dilute acid, alkaline, ammonia, sulphite, sodium chlorite, organic, inorganic solvent, SO_2, CO_2 or other chemicals [21,69,70]. The use of sodium sulphite and/or in combination with sodium chlorite is the most efficient delignifying agent for the removal of lignin to enhance the surface area of the substrate accessible to enzymatic hydrolysis [69]. Alkali pretreatment is also a potential process to remove lignin and uronic acid which decrease the accessibility of enzyme to the hemicellulose and cellulose [6,71]. Sodium, potassium, calcium, ammonium carbonate [50] and ammonium hydroxide are appropriate chemicals for pretreatment. Among these, NaOH has been studied the most [72]. Alkaline peroxide was used for pretreatment of lignocellulosic biomass. This method can enhance the enzymatic hydrolysis by delignification [59]. Organo-solvent provides treated cellulose for easier enzymatic hydrolysis. This method uses an aqueous organic solvent to remove or degrade the complex structure of lignin and hemicellulose [73]. To increase bioethanol productivity with a few inhibitors generated, an efficient and attractive process of combined alkaline peroxide pretreatment and semi-simultaneous saccharification and fermentation (SSSF) was developed. Pretreatment with 10% of H_2O_2 at 160ºC for 2h followed by SSF was found to be effective by achieving ethanol yield about 63.1% [64]. The treatment of lignocellulosic biomass with ozone, referred to as “ozonolysis” can efficiently remove lignin and part of hemicellulose. This pretreatment is generally carried out at room temperature and does not offer the formation of inhibitory compounds [74].

Dilute acid pretreatment

Dilute acid pretreatment is the oldest technology and widely used for converting cellulosic biomass to bioethanol. The method is highly effective due to high reaction rate, thereby achieves a high yield of hemicellulose and significantly increases the availability of cellulose fraction for saccharification [75]. The pretreatment of cotton gin waste with dilute acid is reported to efficiently improve enzymatic hydrolysis [5,46]. Sulfuric acid is the most widely applied acid though other acids such as nitric acid, phosphoric acid, organic acid and HCl were also reported for pretreatment of lignocellulosic biomass [7,8,59,69,70]. However, acid treatment has several disadvantage such as: hazardous,

production of inhibitors (furfural, 5-hydroxymethyl furfural (HMF), weak acids and phenol [8,76] that an adverse impact on the growth of yeast in the fermentation process resulting a decrease in bioethanol yield [5,76]. Furthermore, pretreatment using sulfuric acid involves the formation of large amount of gypsum, which can affect the downstream process and low-value by-products [77]. In this context, the pretreatment of cotton gin waste using organic acid may be more attractive and effective due to less toxic byproduct formation, environmental friendly and commercially available in compared to other conventional acid. However, not much work has been done in this area of research for bioethanol production from cotton gin waste using organic acid. The acid treatment is carried out under low temperatures are optimal to reduce the formation of inhibitors such as hydroxymethyl furfural and to minimize sugar degradation [16,78]. The pretreatment time is dependent on the temperature used, where higher temperatures require shorter reaction times. The use of concentrated acid in the pretreatment is not cost effective and feasible due to corrosion and subsequent toxicity to microorganisms for bioethanol fermentation because of the formation of inhibitory compounds [36]. In addition, the acids must be recovered after the process to make the process economically viable [8,30]. Pretreatment of cotton gin waste with dilute acid can efficiently improve enzymatic hydrolysis [5,46]. Dilute acid hydrolysis occurs in two stages to take advantage of the differences between hemicellulose and cellulose. The first-stage is conducted under mild process condition to recover five-carbon sugars while in second stage only the remaining solids with more resistant cellulose undergo several treatments(biological or chemical) to recover the six-carbon sugars [79].

2.2.4 Detoxification

During acid pretreatment the depolymerization of hemicellulose yields xylose as the major fraction in comparison to other acid pretreatment. However, this method offers some disadvantage like producing toxic inhibitors [69,80,81]. These toxic by-products are divided into three major groups, i.e. organic acids (levulinic, acetic, and formic acids), derivatives of furan (furfural and 5-hydroxymethylfurfural) and phenolic compounds. These inhibitors have an adverse impact on the physiology of yeast cell which results in decreased bioethanol yield and productivity [69,82]. Various methods have been investigated for the removal of fermentation inhibitory compounds like overliming C[82], ethyl acetate extraction [83], activated charcoal adsorption [84] and laccase oxidation treatment [82]. Among the various detoxification methods, overliming and activated

charcoal adsorption methods are most widely used either individually or in combination [69,70].The detoxification of hydrolysates by activated charcoal, is reported as a cost effective with high capacity to absorb compounds without affecting levels of sugar in hydrolysate [69,82].

2.2.5 Biological pretreatment

Biological pretreatment involves microorganisms such as white, brown and soft-rot fungi that are used to degrade or decompose complex lignin and solubilize hemicellulose. White-rot fungi are reported to be the most efficient microbes for delignification of lignocellulosic biomass [30,35,59]. The biological processes using fungal strains are the most attractive for the conversion of this waste to bioethanol. Biological pretreatment using various potential fungal and bacterial strains for the conversion of lignocellulosic biomass to bioethanol is a cost-effective and environmentally friendly process. Whereas the conventional process requires high temperature, pressure and energy for their analysis and corrosion formation are another major drawbacks [85,86]. Biological pretreatment using fungal treatment utilizes their enzyme systems to degrade lignin and hemicellulose compound of lignocellulosic biomass in comparatively low energy, offers minimal byproduct formation, the absence of substrate loss usually occurs due to chemical modification and requires mild environmental conditions [1]. Most of the mixed cultures of white rot fungi were reported for biodegradation in producing high activity enzymes due to their synergistic actions [49,86]. Mixed fungal cultures could lead to a higher enzyme production through synergistic interactions, but the final results seem to depend on several factors such as particular species combination or mode of interaction among species, micro-environmental or nutritional conditions in the substrate under colonization [87]. The most widely studied white-rot fungus is *P. chrysosporium*, which is one of the holobasidiomycetes [75]. The influence of fungus treatment on the biochemical composition and degradation of cotton plant by-products (cotton burns and cotton gin trash) by *Pleurotus sajor caju* were evaluated for lignin degradation [88]. Biodegradation of cotton stalks and cotton seed hull by the oyster mushroom, *Pleurotus ostreatus* was studied for higher yield of bioethanol [89]. Earlier it was reported that some agro-industrial and forestry by-products were subjected to solid-state fermentation by using *Agrocybe cylindracea* and *Pleurotus ostreatus*, where the process and end-products were comparatively evaluated for bioethanol production [90]. Lignin biodegradation by white-rot fungi is an oxidative process and phenol oxidases are the key enzymes [91].

Degradation of lignin by white-rot fungi is the most effective microorganisms for biological pretreatment that occurs through the action of lignin-degrading enzymes such as peroxidases and laccases [35,92]. Some of the enzymes are there, whose roles have not been fully elucidated including glyoxal oxidase, glucose oxidase, oxido-reductase and methanol oxidase [93]. Two groups of peroxidases, lignin peroxidases (LiPs) and manganese-dependent peroxidases (MnPs), have been well-characterized. Laccase enzyme was also well demonstrated in fungi for delignification [94]. Recently some bacterial laccases have also been characterized from *Azospirillum lipoferum* and *Bacillus subtilis* [94]. Several white-rot fungi such as *Phanerochaete chrysosporium, Ceriporia lacerata, Pycnoporus cinnarbarinus, Trametes pubes*cens, *Cyathus stercolerus, Ceriporiopsis subvermispora and Pleurotus ostreaus* have been examined on different lignocellulosic biomass and showed high delignification efficiency [41,72,95,96]. The biological pretreatment might be used for the removal of specific components such as antimicrobial substances and detoxification to improve its digestion [59]. Biological delignification processes are being developed for their integration in biomass to bioethanol process. Solid and submerge state of cultivation are the method of choice for biological delignification. Solid-state fermentation is a efficient and provides a suitable cultivation environment for delignification of lignocellulosic biomass [97]. *Pycnoporus cinnabarinus* fungus was compared with commercial enzyme laccases from *Trametes villosa* and *Myceliophthora thermophila* in terms of stability and mediator oxidation rates [92]. *Rigidoporous ligno-sus*, a white-rot basidiomycete excreted two oxidative enzymes into the culture medium: laccase and Mn peroxidise, and these two enzymes acted synergistically in solubilizing the lignin [98]. Wang et al. 1990 first cloned a lignin peroxidase gene from *Streptomyces* v*iridosporus* T7A into *Streptomyces lividans* and demonstrated that the genetically engineered *S. lividans* expressed significant extracellular 2, 4-dichlorophenol peroxidase activity and degraded lignocellulose in solid state processes [61]. Most lignolytic, microorganisms solubilize or consume not only lignin but also hemicellulose and cellulose [99]. Cultivation of edible mushrooms such as *Lentinula* spp, *Lentinus* spp, *Agaricus* spp, *Leonotis* spp, *Volvariella* spp, *Pleurotus* spp, *Lentinus* spp, *Agrocybe* spp, and *Grifola* spp are achievable on a wide range of lignocellulosic waste. Several newly isolated microorganisms were also explored to enhance the delignification process [9,100].

2.6 Hydrolysis

This treatment of biomass is carried out by the breakdown of carbohydrate polymers to free sugar monomers. This method is known as hydrolysis as the process involves the addition of one water molecule for every glycosidic bond broken. Hydrolysis methods include an enzymatic method (fungal or commercial enzyme), steam explosion, dilute and concentrated acid methods for lignocellulosic biomass have been reported. Among these, acid hydrolysis and enzymatic hydrolysis are mostly used [18,68]. In acid hydrolysis, a little amount of dilute/concentrated non-oxidising acid like HCl or H_2SO_4 is used to release monomer sugars from biomass. In this process, acid acts as a catalyst by providing H^+ ions to facilitate the intake of H_2O molecules. On the other hand, enzymatic hydrolysis is also suitable options for the conversion of polysaccharide to monosaccharide by avoiding toxic by-product formation. Cellulase, the most commonly used enzymes for depolymerization of cellulose to glucose, consists of three major classes of enzymes namely exoglucanases, endoglucanases and β-glucosidases [101]. The endoglucanases catalyze the irregular cleavage of internal bonds of the cellulose polymer chain, when cellobiohydrolases attack the ends of the chain by releasing cellobiose but β-glucosidases attack on cello-oligosaccharides and cellobiose, thereby release glucose monomers units from the cellobiose. A variety of microorganisms including fungi and bacteria was reported to degrade cellulosic biomass to monomer glucose. Wood et al. (1986) isolated an anaerobic fungus, *Neocallimastix frontalis* from the rumen of a sheep which produced a highly active extracellular cellulase for the release of monomer sugars from cotton fiber. The cellulose was several-fold more active in solubilising cotton fiber per unit of endo-1,4-b-glucanase than the cellulase of the aerobic fungus *T. reesei* mutant strain C-30, which is one of the most active cellulases isolated so far [102]. Due to the complex structure of pentose or hemicellulose, several different enzymes are needed for their enzymatic degradation. The two main glycosyl hydrolases depolymerizing the hemicellulose backbone are endo-1, 4-β-D-mannanase and endo-1, 4- β-D-xylanase [103]. Cellulase enzyme production from *T. reesei* 3EMS35 mutant hydrolyzed most of the cellulose (91 %) in wheat straw which is further used for higher bioethanol production [104]. The strains of *Trichoderma, Aspergillus, Penicillum and Altrernaria* were isolated from different animal dung, manure and soils are reported to have highly lingnocellulolytic activity that is cellulolytic activity along with lignolytic activity and hemicellulolytic activity [105]. Some of the chemical and biological surfactants such as

Tween 20, Tween 80, PBS and PEG have been reported to facilitate the conversion of cellulose thereby an enhanced yield of sugar by enzymatic hydrolysis was achieved [59,70].

2.7 Fermentation

In this section, an overview of the current status of bioethanol fermentation using potential microorganisms and different technology and conditions implied for efficiency of conversion. Recently, *Kluyveromyces marxianus* has become a species of researcher's interest for bioethanol production at high temperature from a wide variety of substrates. However, the reason behind the production of bioethanol by this yeast at high temperature is unknown [106]. *H. polymorpha* ferments both glucose and xylose up to 45°C [107]. *Debaryomyces sp* is a thermotolerant organism which used for both pentose and hexose fermentation with a preference for one carbohydrate does not inhibit the consumption of other [103]. *Neurospora crassa* is efficient to produce bioethanol directly from the cellulose/hemicellulose, since it produces both the cellulase and xylanase and also has the capacity to ferment the sugars to bioethanol anaerobically [108–110]. Some of genetically engineered strains were reported for the production of bioethanol from sugars with high efficiency by utilizing the *Saccharomyces, Pichia stipitis* and *Zymomonas mobilis* strains tested under RaBIT fermentations to determine their suitability for this platform [111]. An efficient conversion of glucose and xylose of lignocellulosic biomass was fermented with an innovative designed scheme involving co-culturing of *Zymomonas mobilis* and *Pichia stipitis* in a modified fermentor [112]. The gram-negative bacteria *Klebsiella oxytoca, Z. mobilis pdc* and *adhB (*recombinant derivatives) genes have been integrated into the chromosome for directing the metabolism of pyruvate to bioethanol, where the rates of ethanol production of this recombinant strain were estimated at temperature 37°C and pH 5.0 under different stresses [113]. Whereas, co-culturing of different strains together seems to be very promising for the high-level production of ethanol with minimizing the inhibitory effect of cellobiose [114,115]. Engineered *Saccharomyces cerevisiae* was reported as the most effective strains for higher yielding of ethanol production [116]. The microaeration process enhanced the productivity of ethanol from biomass using ethanologenic *E. coli* [117], simultaneous saccharification and fermentation (SSF) using recombinant *Saccharomyces cereviasiae* resulted high ethanol production [118]. The saccharification of the lignocellulosic biomass by the enzymes and the subsequent

fermentation of the sugars to ethanol by the yeast *Saccharomyces, Zymomonas* mixed with *Kluyveromyces fragilis* have produced improved ethanol production [119]. The main advantage of using SSF for the ethanol bioconversion is the enhanced rate of hydrolysis of lignocellulosic biomass (cellulose and hemicellulose) due to the removal of end product inhibition. Separated hydrolysis and fermentation is a conventional two-steps process where the hemicellulose is hydrolyzed using the enzymes to form the reducing sugars in the first step and subsequent fermentation of sugars, thus formed, are fermented to the ethanol in the second step. The advantage of this process is that each step can be carried out at its optimum conditions. Different protoplasts fusant strains were also reported as *Saccharomyces cerevisiae*, glucose fermenting yeast and *Pachysolen tannophilus, Pichia stipitis* and *Candida shehatae* as xylose-fermenting yeasts. The fusants were used for fermentation of glucose-xylose mixture and the highest ethanol producing fusant was used for the further study to ferment hydrolysates produced by acid pretreatment and enzymatic hydrolysis of cotton gin waste [5,120]. *Escherichia coli* KO11 was reported as genetically engineered bacteria to produce ethanol from pentose and hexose sugars by inserting genes encoding alcohol dehydrogenase and pyruvate decarboxylase from the bacterium *Zymomonas mobilis*. Efficient *E. coli* KO11 was reported to metabolize complex mixtures of sugars [121]. Kim. *et al*., found that the fermentation of glucose and xylose attained a level of 90% ethanol production at 12h using *S. cerevisiae* K35 and *P. stipitis* KCCM 12009 [122]. Lu Y et al. reported that the improvement of robustness and ethanol production is more of ethanologenic *Saccharomyces cerevisiae* under co-stress of heat and inhibitors [123]. Whereas, some yeast strains are enriched in aeration condition and some are not. Due to these problems some modification in aeration condition was done to enhance bioethanol production using sequential use of yeast strains in a bioreactor [124].

2.8 Conclusion

In summarizing the literature reviews, the advantages of fuel bioethanol from lignocellulosic biomass is a potential energy source. Waste biomass is one of the richest carbon sources and requires innovative major conversion technologies such as pretreatment, hydrolysis and fermentation which must be cost-effective and environmental-friendly.

Chapter 3

Scope and Objectives

3 Scope and Objective

In recent years, bioethanol is considered as a promising alternative energy source for the transportation sector. Lignocellulosic biomass is the most attractive feedstock for the production of fuel bioethanol because of its widespread availability, renewable resource and cost effective. A huge quantity of cotton gin waste generated in cotton industry is considered as a potential lignocellulosic biomass for the production of bioethanol. However, two major challenges lie in its conversion to bioethanol-(i) effective pretreatment for delignification thereby release of cellulose and hemicelluloses for efficient hydrolysis (ii) a suitable microbial system for the conversion of C5 and C6 sugars by fermentation. Keeping this in view, the present research has been undertaken with the aim of developing an effective pretreatment process and suitable microbial system for fermentation to produce bioethanol from cotton gin waste.

The main **objectives** of the present research work are as follows:

I. To characterize the chemical composition of cotton gin waste
II. To develop an efficient and environment-friendly process for the pretreatment of cotton gin waste
III. To evaluate the performance of enzymatic hydrolysis of pretreated cotton gin waste to maximize C5 and C6 sugar
IV. To optimize the conversion of pentose and hexose sugars to bioethanol

The scope of the research work:

The present investigation focuses on a systematic research towards efficient pretreatment, enzymatic hydrolysis and fermentation for the production of bioethanol from industrial cotton gin waste. The whole research work has been divided into the following parts:

- **Composition analysis of cotton gin waste**

The composition of any lignocellulosic biomass varies according to their season of harvesting and land quality. In this part of the study, the composition of cotton gin waste was analyzed in terms of carbohydrates, ultimate (elemental) and proximate (ash and moisture) analysis.

- **Collection, isolation and identification of fungi**

In this area of research, four commercially available white rot fungal strains namely, *Trametes pubscens, Pleurotus ostreatus, Pycnoporus cinnabarinus* and *Phanerochaete chrysosporium* were used for the delignification of cotton gin waste. An effort has also been given to isolate fungal strains from the soil of a dumping area of a cotton ginning mill. The isolated fungal strains were evaluated for their efficiency for lignin degradation. The phenotypic characterization of unknown fungi was identified.

- **Pretreatment of cotton gin waste**

This is one of the major areas of the present investigation. The present study investigated the effect of organic acids pretreatment for the conversion of hemicellulose to C5 sugars due to their natural availability and non-hazardous chemical properties in comparison to conventional and widely used dilute sulfuric acid pretreatment. It is further evident from the literature that biological pretreatment using white rot fungi is an attractive conversion technique for delignification thereby facilitating hydrolysis and fermentation for bioethanol production. Therefore, in this study, the effect of fungal pretreatment using *Trametes pubscens, Pleurotus ostreatus, Pycnoporus cinnabarinus* and *Phanerochaete chrysosporium* individually and co-culture on delignification of cotton gin waste was investigated by the solid and submerged state of cultivation. Pretreatment was also performed using newly isolated fungal strains from the soil of a dumping area of cotton waste. Furthermore, the effect of washing, heating and detoxification as the post treatment steps were investigated to asses of their any impact on enzymatic hydrolysis.

- **Optimization of pretreatment parameters by response surface methodology**

The influence of temperature, pH and shaking speed on the pretreatment of cotton gin waste was investigated to determine the optimum pretreatment condition through response surface model using the central composite design for delignification of biomass thereby facilitating the release of cellulose and hemicelluloses in solid and submerge state of cultivation.

- **Hydrolysis of C5 and C6 sugars**

In this phase of research work, the efficiency of pretreatment using organic acids as well as fungal strains was evaluated in terms of release of pentose and hexose sugar by enzymatic hydrolysis. The hydrolysis of pretreated biomass was carried out using commercial enzymes such as cellulase, xylanase and β-gucosidase.

- **Fermentation of hydrolysates (C5 and C6 sugars) to produce bioethanol**

The fermentation experiments were performed for the conversion of acid and enzymatic hydrolysates derived from pretreated cotton gin waste. An effort has been given for the improvement of C5 and C6 sugar conversion by fermentation by investigating the efficiency of the individual, co-culture and sequential use of yeast strains. The fermentation process was also studied elaborately to maximize the sugar conversion, ethanol yield and ethanol productivity. An attempt has been given for large-scale bioethanol production by conducting fermentation experiment in a bench top bioreactor.

Chapter 4

Materials and Methods

4 Materials and Methods

4.1 Biomass collections, processing and composition analysis

Cotton gin waste (CGW) was collected from Shree Ambica Agro Industries Ltd., Balangir, Orissa, India. The biomass was washed thoroughly and dried in an oven for 48h at 60ºC. The waste containing lengthy cotton fibers was subjected to reduce its length in the range 0.2 to 0.5 mm by milling (pulverisette-5 machine, Fritsch Company). The biomass was oven dried for 6h at 60ºC to remove left over moisture.

4.1.1 Composition analysis

The biomass was extracted with alcohol-benzene (1:2 volume ratios) mixture for 4h using Soxhlet extraction apparatus and extractive content were estimated. The chemical composition of the extractive-free biomass was analyzed. The analysis of carbohydrate fractions of cotton gin waste was estimated following the TAPPI protocol of chemical analysis. All the experiments were performed in triplicates and the values were presented as mean.

Moisture content

The moisture content of biomass was analyzed by drying 1gm sample in a silica crucible at 105 ºC in a hot air oven till a consistency in weight was observed. The moisture content was calculated by dry and wet basis as follows:

Moisture (wet & dry basis) = $(W_2 – W_f) / (W_2 – W_1) \times 100$

Where, W_1 is the weight of empty crucible
W_2 is the weight of the crucible and sample
W_f is the constant weight of crucible and sample after drying

Ash content

The biomass was taken in a pre-weighed silica crucible and combusted in a muffle furnace at 750 ± 25ºC for about 4h.

The ash content was estimated using following formula:

% Ash (dry basis) = $(W_f - W_1) / (W_2 - W_1) \times 100$

Where, W_1 and W_2 are the weight of the silica crucible and oven-dried sample, and W_f is the final weight of crucible containing the sample after combustion.

Elementary analysis

The elementary analysis including carbon (C), nitrogen (N) and hydrogen (H) present in cotton gin waste was done by CHNSO analyzer (PerkinElmer 2400) following standard protocol (ASTM 5373). The analysis was carried out by 1.0 mg of biomass was heated in a tin boat assortment at 900°C in an oxygen atmosphere where carbon is converted to carbon dioxide, nitrogen (N) to nitrogen gas (N_2) and hydrogen to H_2O. The oxygen content was determined by means of difference.

Cellulose

10 ml acetic acid/nitric acid solution (15 ml of 80% acetic acid mixed with 1.5 ml of conc. nitric acid) was added to 0.1g cotton gin waste and the mixture was kept in a water bath for 30 min. After cooling, the biomass slurry was centrifuged at 8000 rpm for 10 min and supernatant was discarded. The sample pellet was washed thoroughly with distilled water and then 10 ml sulfuric acid (67% v/v) was added. The resulting suspension was allowed to stand for 1h followed by addition of 4 ml anthrone reagent to 1 ml diluted mixture. The mixture was incubated in a water bath for 15 min for color development. After cooling, the optical density was measured by spectrophotometers at 620 nm against the reagent blank (1ml distilled water and 4ml anthrone reagent). The percentage of cellulose was calculated from glucose standard curve by the following formula:

$$\%\ \mathbf{Cellulose} = \frac{\mathbf{glucose\ released\ (g)} \times \mathbf{0.9}}{\mathbf{Sample\ dry\ weight\ (g)}} \times \mathbf{100}$$

Where, 0.9 is the correction coefficient for hydration [125]

Hemicellulose

For the analysis of hemicellulose, 10 ml of 3% (w/v) sulfuric acid was added to a 1 g oven-dried biomass sample and then autoclaved at 121ºC for 15 min with pH 7.0 which was adjusted with potassium hydroxide and hydrochloric acid. The sample was diluted 10 times with distilled water and 1 ml p-bromoaniline reagent was added to 5 ml diluted sample. The suspension of the sample was kept in water bath at 70ºC for 10 min followed by incubation in a dark chamber at room temperature for 70 min. The optical density of the sample was measured at 520 nm against reagent blank. The percentage of hemicellulose was calculated using xylose and arabinose standard curves. The hemicellulose content was calculated using below equation as follow:

$$\%\ \mathbf{Hemicellulose} = \frac{(\mathbf{Xylose\ +\ arabinose\ released})\ (\mathbf{g})\ \times\ \mathbf{0.9}}{\mathbf{Sample\ dry\ weight}\ (\mathbf{g})}\ \times \mathbf{100}$$

where , 0.9 is the correction coefficient for hydration [125]

Lignin

1 gm oven dried extractive free waste was treated with 15 ml of 72% (w/v) sulfuric acid for 2h at 20ºC with occasional stirring. The sample was transferred to a flask and acid concentration was brought down by adding distilled water (560 ml) with refluxing for 4hr. The contents were filtered through G3 crucible and washed with distilled water until free from acid. Further, the crucible containing a biomass was dried to constant weight at 105ºC in an oven. The lignin content of biomass was determined by the following equation:

% Lignin = (B/A) × 100

A = Initial weight of oven dried sample

B = Final weight of oven dried lignin

Estimation of carbohydrates

The monomer sugars of raw biomass were analyzed using high-performance liquid chromatography (HPLC) (Agilent Technologies 1260 Infinity) with an RI detector for the estimation of the carbohydrates (glucose, xylose and arabinose). Hi-Plex H column was used with 0.005 M H_2SO_4 as an eluent at a flow rate of 0.7 ml/min and 60ºC.

4.1.2 Media and buffers

The media and buffers used in different conversion processes, as well as the experimental analysis are listed in Tables 1 and 2.

Table 1: Media composition

Sl. No	Media	Composition	pH
1	YPD Agar for yeast growth	10 g/l Yeast extract, 20 g/l peptone, 15 g/l Agar	pH 6.5
2	YPD Broth	10 g/l Yeast extract, 20 g/l peptone, 20 g/l Agar	pH 6.5
3	PDA broth for fungal growth	Himedia- Potato dextrose broth 24 g/l	pH 5.6
4	PDA agar	Himedia- Potato dextrose broth 39 g/l	pH 5.1
5	Fermentation media	2 g/l yeast extract, 0.4 g/l $MgSO_4$, 2 g/l $(NH_4)SO_4$, 5 g/l KH_2PO_4 and 200 g/l glucose and xylose mixture (3:1 Ratio).	pH 4.5

Table 2: Buffers and their composition

Sl.No.	Buffer	Composition	pH
1	20 mM Acetate buffer	0.1M Acetic acid and 0.1 M sodium acetate	4.5
2	50 mM citrate buffer	0.1 M citric acid (24.3 ml/100 ml) and 0.2 M dibasic sodium phosphate(25.7 ml/100 ml)	5

4.2 Pretreatment

4.2.1 Organic acid pretreatment

Four organic acids such as oxalic, citric, acetic, maleic acid and one most widely used sulfuric (inorganic) acid were used for the pretreatment of cotton gin waste. The pretreatment experiment was carried out at 150°C, 45 min, 10% (w/w) biomass loading and 500mM acid concentration in the 250 ml conical flask. The dilute acid pretreatment process was performed in a hot air oven to maintain a constant temperature. After pretreatment, the hydrolysate was filtered through vacuum filtration for the analysis of released sugar and the biomass was washed properly with running tap water until a neutral pH was achieved. All the experiments were performed in triplicates and the values are presented as mean.

The combined severity factor was calculated by the relation described by Chum et al. 1990 [126].

$$Log[t.exp(\frac{T_p - T_{ref}}{14.75})] - pH$$

Where t is the time(150°C), Tp (45 min) and T_{ref} are pretreatment and reference temperature which usually set to 100°C [7]. The pH was measured before the pretreatment.

4.2.1.1 Optimization of pretreatment condition

The pretreatment experiments were carried out at different temperatures (100, 130 &150°C), time periods (30, 45 & 60 min) and concentration of acids (300, 500 & 700 mM) to establish the optimum pretreatment condition. The solid loading concentration was kept constant at10% (w/v). The hydrolysate was recovered by filtration through a double-layered muslin cloth and used for sugar analysis. The residual biomass was washed thoroughly and dried overnight at 60°C for further delignification.

4.2.1.2 Detoxification

The overliming and activated charcoal adsorption method was followed for detoxification [127]. The detoxification of acid hydrolysate was analyzed by overliming and activated charcoal treatment, both in combination and individually. The acid hydrolysate of waste

biomass was treated with calcium hydroxide to increase its pH up to 10. The total slurry was stirred for 30 min and cooled to room temperature. The hydrolysate was neutralized and centrifuged at 10,000g for 15 min. The overlimed acid hydrolysate was treated with 1.5% (w/v) activated charcoal with continuous stirring (60 min) at room temperature and the resulting sugar was separated by vacuum filtration [69].

4.2.1.3 Delignification of acid pretreated biomass

The acid pretreated biomass was delignified according to the method described by Kuhad et al. [69]. The acid-pretreated cotton gin waste was treated with a mixture of 5% (w/v) sodium sulphite and 3% (w/v) sodium chlorite at different temperatures (100-140°C) and time intervals (30-60 min). The solid loading of biomass was 10% (w/v).The delignified biomass was then filtered through double-layered muslin cloth and the cellulosic residue was washed thoroughly with tap water until a neutral pH was achieved. The biomass was then dried overnight at 60°C and percentage of lignin removal was calculated from the delignified biomass.

4.2.2 Biological pretreatment

4.2.2.1 Organisms

Trametes pubscens (NCIM.No-1087), *Pleurotus ostreatus* (NCIM.No-1200), *Pycnoporus cinnabarinus* (NCIM.No-1181) and *Phanerochaete chrysosporium* (NCIM.No-1197) were collected from National Collection of Industrial Microorganisms, Pune, India. Fungal strains were inoculated on potato dextrose agar (PDA) plates and incubated for 4-5 days at 35ºC and finally stored in the refrigerator for further use.

4.2.2.2 Isolation and screening of fungal strains

Fungal strains were also collected from the soil of dumping site of the cotton gin waste of Shree Ambika Agro Cotton mill, Orissa, India. Fungal strains were separated using serial dilution of collected sample and culture on potato dextrose agar (PDA) plates for incubation 4-5 days at 35ºC. The fresh pure strains were finally stored in the refrigerator for further analysis. Selection of effective ligninolytic fungi was done by using 0.1 ml of fresh fungal mycelium from the enriched culture medium containing malt extract agar, supplemented with 0.02% Guaiacol and 10-25 µg/ml of Tetracycline at pH of 6.0 and incubated at 28±1°C for 5-7 days [128]. The efficiency of fungal growth with respect to

enzymatic activity was measured by the reddish brown colour circular zone of activity which developed due to the oxidation of guaiacol and noted as ligninase positive.

4.2.2.3 Identification of isolated fungi

The phenotypic characterization of unknown fungal strain was done at Institute of Microbial Technology (IMTECH), Chandigarh, India. Further, the structural identification of fungi was observed under light microscopy. The spore structure of the fungi was identified by lactophenol cotton blue mounting method [129].

4.2.2.4 Biological Pretreatment of cotton gin waste

The pretreatment of cotton gin waste was carried out by submerge (SMC) and solid state (SSC) cultivation. In SMC pretreatments, 6g air dried cotton waste was supplemented with 108 ml acetate buffer (20mM, pH 4.5) and 1ml spore inoculum. For SSC pretreatment, 6g the cotton gin waste was mixed with 9.6 ml acetate buffer (20mM, pH 4.5) and 6ml spore inoculums to obtain 75% substrate moisture content (wet basis). Sample without fungal strain was used as a control. The fungal pretreatment experiments were carried out in 250ml Erlenmeyer flasks were capped with a silicon stopper with inlet and exit lines connected to 0.2 μm filters. Flasks with cotton waste were autoclaved for 20 mins at 121^{o}C and 15psi, cooled, mixed with acetate buffer, and then inoculated with spore suspension ($5x10^{6}$ spores g^{-1} cotton waste). After adequate growth Tween 80 (0.25%) surfactant was added to increase the laccase activity in pretreatment process [130]. Pretreatments were performed in air convection incubator at 35^{o}C with a shaking speed of 100 rpm, and flasks were flushed with oxygen for 10 min in every 7days, starting from day 0 to 40 days of the experiment. Both SMC and SSC cultivated flasks were sampled on every 8 days and stored at 4^{o}C for composition analysis.

4.2.2.5 Optimization of pretreatment parameters

A three level RSM based on central composite design (CCD) was employed for the optimization of pretreatment process using Minitab 16.2v software, involving 20 combinations with 6 center point of three variables. Statistical analysis was performed with 95% confidence level. Three different parameters (independent variables) selected for this study are (i) pH at three levels 4, 4.5 and 5, (ii) temperature 30, 35 and 40^{o}C, and (iii) rpm 100, 120 and 140. The optimization of pretreatment process has been conducted for 32 days of using solid state cultivation and the pretreatment experiments were carried

out in triplicates. In coded terms, the lowest, middle and the highest level of 3 variables were –1, 0 and +1 respectively. Table 3 represents the coded and actual values of the factor levels used in the experiments. After 32 days of incubation, the total lignin content of untreated cotton gin waste was determined.

Table 3: Independent variables and their corresponding levels used in RSM study

Factors	Coded unit	−1	0	+1
pH	A_1	4	4.5	5
Temperature(^{o}C)	A_2	30	35	40
RPM	A_3	100	120	140

4.2.2.6 Wash and heat wash pre-hydrolysis treatments

The pretreated samples were washed three times with distilled water and autoclaved for 10 min at 121^{o}C and15 psi followed by washing. These treatments were carried out after 32 days of the solid state of cultivation. Wash and heat-wash treatments were tested to remove fungal biomass as well as inactivation of fungus [131].

4.2.3 FTIR, XRD and SEM analysis of untreated and pretreated cotton gin waste

FTIR spectra of dried cotton gin waste samples were recorded on FTIR spectrophotometer (Perkin Elmer-Version 5.3). The samples were mixed with KBr for their uniform dispersion and spectra were obtained over the range of 400-4000 cm^{-1} with a spectral resolution of 0.5 cm^{-1}.

The overall crystallinity of untreated and pretreated samples was measured by XRD study (XRD PW 3040) using Cu Kα radiation (α = 1.54 Å) at 30 kV and 20mA. The samples were scanned and intensity was observed at 2θ range from 20° to 70° with a scanning speed of 3°/min. Crystallinity was calculated as per the formula $[(I_{002} - I_{am})/I_{002}] \times 100$, where I_{002} represents maximum crystalline intensity peak at 2θ between 22° and 23° for cellulose I, and I_{am} represents minimum crystalline intensity peak at 2θ between 18° and 19° for cellulose I [132,133].

SEM images of untreated and pretreated samples of cotton gin waste were obtained after drying followed by coating with platinum using JEOL JSM6480 LV SEM. The powdered biomass was mounted on a conductive tape and coated with gold palladium. The SEM

images were captured at 10-20 KV at different magnification ranging from 200x to 5000x depending on the image structure.

4.3 Enzymatic hydrolysis

4.3.1 Enzymatic hydrolysis for the acid pretreated biomass

The enzymatic hydrolysis of delignified biomass was performed accordingly to the method reported earlier [70].The enzymatic hydrolysis was performed in a 500 ml Erlenmeyer flasks using enzyme β-glucosidase obtained from *Aspergillus niger* (Novozyme 188) and cellulase from *Trichoderma reesei* (ATCC 26921) from Sigma-Aldrich. The delignified cellulosic biomass 5% (w/v) was suspended in 0.05 M citrate phosphate buffer (pH 5.0) at 50ºC and soaked in a shaking incubator for 2h. The delignified cellulosic biomass was suspended in 0.05 M citrate phosphate buffer (pH 5.0) at 50°C and soaked in a shaking incubator for 2h. The suspension was further supplemented with cellulase (3 FPU/ml) and β-glucosidase (Novozyme 188) (9FPU/ml). Enzymatic hydrolysis was performed at 50°C and 150 rpm for 40h. A dose of 0.005% sodium azide was added to avoid microbial contamination and 1% (v/v) Tween 80 with 0.25% bovine serum albumin (BSA) was introduced for better accessibility of enzymatic action [70]. Samples were collected every 4h for analysis of sugar.

4.3.2 Enzymatic hydrolysis of the biologically pretreated biomass

Enzymatic hydrolysis of biologically pretreated cotton gin waste was performed following the method published earlier at the optimum ratio of xylanase and cellulase (1 : 3) enzymes during the hydrolysis [134]. Biological pretreated biomass was supplemented with cellulase (5mg), xylanase (30mg) and β-glucosidase (15 mg) enzymes using 0.05 M citrate phosphate buffer (pH 5.0). Enzymatic hydrolysis was performed at 50°C and 150 rpm for 64h. A dose of 0.005% sodium azide was added to avoid microbial contamination and 1% of Tween 80 was introduced for better accessibility of enzymatic action. The percentage of cellulose and hemicellulose hydrolysis was decreased by the addition of BSA in hydrolysates using cellulase and xylanase enzyme [134].

4.4. Fermentation

4.4.1 Microorganisms and culture medium

Pichia stipitis ((NCIM 3498) and *Saccharomyces cervisiae* (NCIM. No-3090) were obtained from National Collection of Industrial Microorganism (NCIM), Pune, India. The *S. cervisiae* strain was maintained in medium containing (g/l): glucose, 30; yeast extract, 3; peptone, 5; agar, 20 at pH 6.0 ± 0.2 and temperature 30°C [135]. The medium for *P. stipitis* inoculum was (g/l): xylose, 50.0; yeast extract, 3.0; malt extract, 3.0; peptone, 5.0; at pH 5.0 ± 0.2 and temperature 30°C [135].

4.4.2 Fermentation in shake flask

The mixture of acid and enzymatic hydrolysates were supplemented with (g/l): yeast extract, 3; peptone, KH_2PO_4, 2; $(NH_4)_2HPO_4$, NH_4Cl, $MgSO_4.7H_2O$, 0.5; $MnSO_4.5H_2O$, $CaCl_2.2H_2O$, $FeCl_3.2H_2O$ and $ZnSO_4.7H_2O$, 0.1. Further, the media was autoclaved at 110°C for 20 min and then cool it for fermentation. Initially, the fermentation experiments were conducted in shake flasks with glucose fermenting *S. cerevisiae* and xylose fermenting *P. stipitis* at 30°C with pH 5.5 and 150 rpm for 72h [124]. The fermentation was carried out in different conditions of yeast strains such as co-culture, individual culture and sequential use of yeast strains by adding *S. cerevisiae* initially, after fermentation of glucose (8-24h), the temperature of fermentor was kept at 50°C for 5h for inactivation of *S. cerevisiae* cells and then cooled down to 30°C and *P. stipitis* was added (OD_{600} 3.0). Samples were collected at various intervals and centrifuged at 3000g for 10 min at 4°C and analyzed for residual sugars, bioethanol and growth of strains.

4.4.3 Fermentation in bioreactor

The fermentation of hydrolysates was also carried out in a bench top bioreactor as shown in figure 4.1. Two liters of hydrolysates was mixed and supplemented with fermentation medium (g/l): yeast extract, 3; peptone, KH_2PO_4, 2; $(NH_4)_2HPO_4$, NH_4Cl, $MgSO_4.7H_2O$, 0.5; $MnSO_4.5H_2O$, $CaCl_2.2H_2O$, $FeCl_3.2H_2O$ and $ZnSO_4.7H_2O$, 0.1. The fermentation experiment was conducted in a 5-L laboratory bench top fermentor (Biostat B Plus, Sartorius, India) using 10% inoculum of *S. cerevisiae* and *P. stipitis*. The fermentation was performed with 2L of mixed hydrolysates at different temperature (25, 30, 35 & 40°C), pH (4.5, 5, 5.5 & 6) and agitation speed (150, 200, 250 & 300 rpm) for 64h of

fermentation. Initially, *S. cerevisiae* was added for the fermentation of glucose (8-10h) and then the bioreactor was kept at 50°C for 6h to inactivate the *S. cerevisiae* strains and allow to cool the media for the addition of *P. stipitis* (OD_{600} 6) with aeration of 2 ml/min continuously for 64h. The pH of the medium was adjusted with 2N HCl and 2N NaOH. Samples were taken at regular intervals of 4h and centrifuged at 10,000g for 15 min at 4°C. The cell free supernatant was used to determine the ethanol concentration, residual sugar concentration and biomass yield.

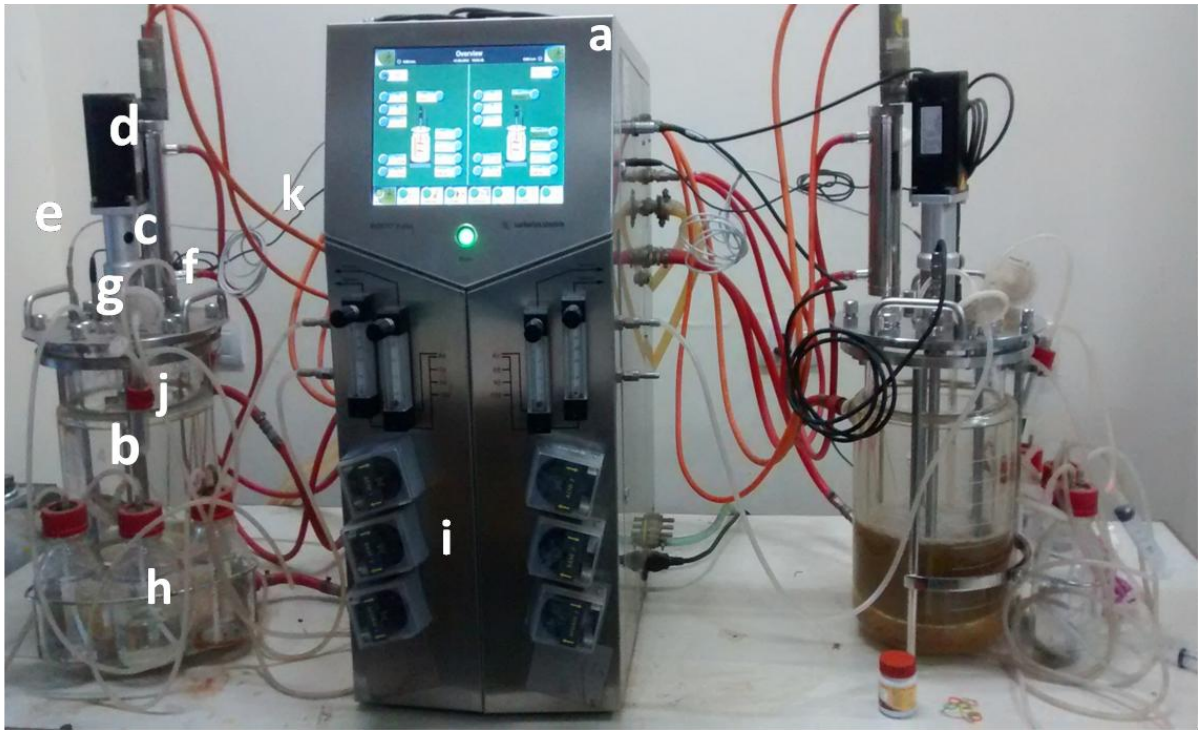


Figure 4.1: Bioreactor setup for ethanol fermentation

Salient features of bioreactor

a) Digital controller
b) 5L scalable glass culture vessel
c) Agitator
d) Agitation motor
e) pH probe
f) DO probe
g) Temperature probe
h) Acid and base
i) Peristaltic pumps
j) Sampling system
k) Gas flow system

4.5 Analytical methods

Ethanol estimation

The ethanol content was determined by gas chromatography (Agilent technology, USA) with an elite-wax (cross bond-polyethylene glycol) column (30.0 m× 0.25 mm) at an oven temperature of 85°C using flame ionization detector (FID) at 200°C. The ethanol standards were prepared using commercial grade ethanol (Sigma-Aldrich). Nitrogen with a flow rate of 0.5 ml/min was used as the carrier gas. The theoretical ethanol yields from glucose were calculated according to the following equation:

The total theoretical ethanol yield efficiency was calculated according to the following equation:

$$\%\ \text{Total ethanol yield efficiency} = \frac{\text{Ethanol produced (g)}}{\text{The amount of glucose and xylose (g)} \times 0.511} \times 100$$

Sugar estimation

Total reducing sugars were estimated by the DNS method and saccharification efficiency was calculated by the following formula [69] :

$$\%\ \text{Saccharification} = \frac{\text{Amount of glucose release (mg)}}{\text{total sugar concentration in the pretreated sample}} \times 100$$

Rate of Enzymatic hydrolysis was calculated as concentration of glucose released per hydrolysis time as per the following equation:

$$v = \frac{ds}{dt} = \frac{Glu_t - Glu_0}{t - t_0}$$

Where,

v = enzyme hydrolysis rate (mg/ml glucose per hour)

$\frac{ds}{dt}$ = amount of substrate concentration consumed per unit time

*Glu*t = Concentration of glucose at time, t (mg/ml),

*Glu*0 = Initial glucose concentration at time = 0h (mg/ml),

t = hydrolysis time (h), and

t_o = time = 0 hour (h)

The estimation of sugars in hydrolysate and biomass was done by NREL protocols using HPLC (Agilent Technologies 1260 Infinity). Hi-Plex H column was used with 0.005 M H_2SO_4 as an eluent at a flow rate of 0.7 ml/min and temperature at 60°C with an RI detector.

Estimation of inhibitor

The total phenolics and total furfural released were determined by spectrophotometric determination following 4 amino antipyrine method [136] and based on the difference in absorbance at 284 and 320 nm after pretreatment [137]. Acetic acid was estimated by HPLC (Agilent Technologies 1260 Infinity).

Biomass estimation

The cell mass concentration was estimated by dry cell mass weight measurement and the pellet obtained by centrifugation of fermentation broth was dried at 70^{o}C till constant weight is achieved. The biomass was also estimated by measuring optical density at 620nm. The total biomass yield of yeast cells was estimated by dry cell mass weight measurement.

Note: The flow charts showing the methodology for the major processing steps involved in the acid and biological pretreatment together with hydrolysis and fermentation for bioethanol production are presented in figure 4.2 and 4.3.

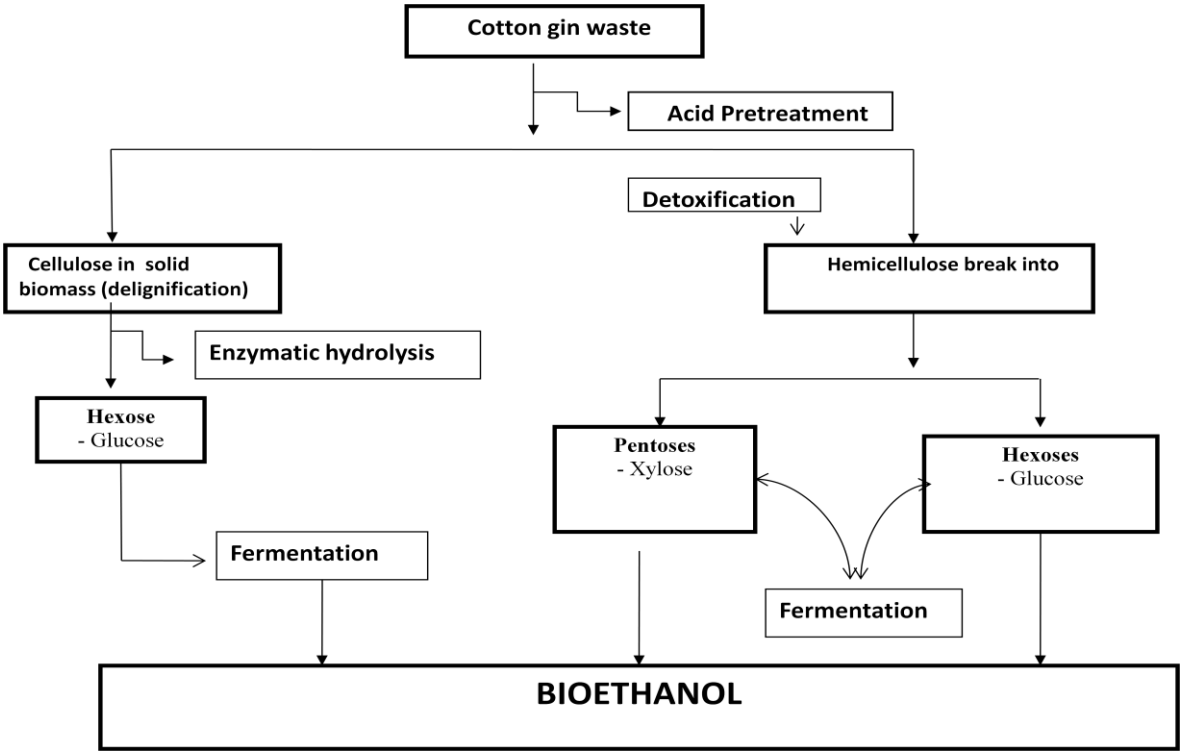


Figure 4.2: Production of bioethanol from cotton gin waste by using fungal pretreatment method

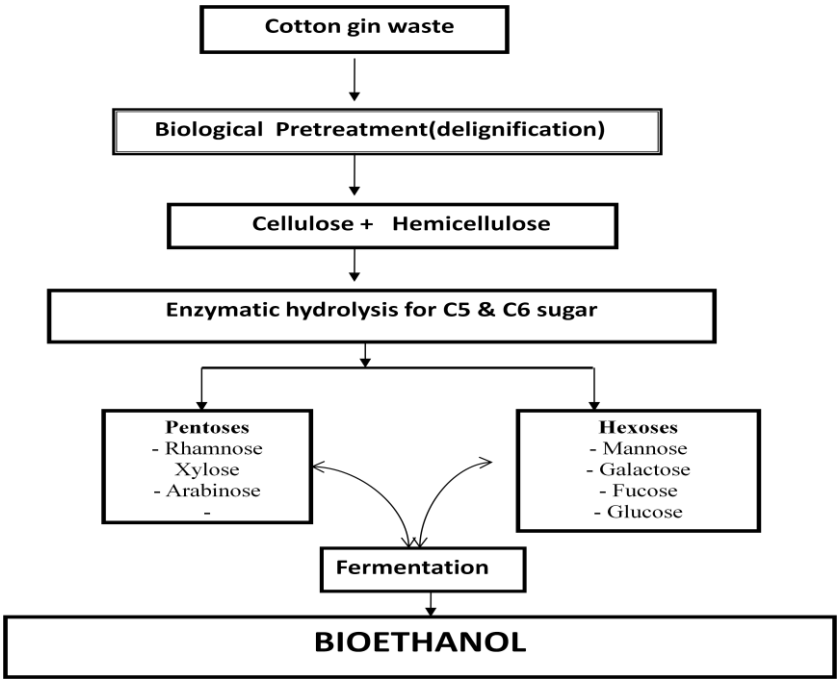


Figure 4.3: Production of bioethanol from cotton gin waste by using fungal pretreatment method

Chapter 5

Results and Discussion

5 Results and Discussion

5.1 Bioethanol production from cotton gin waste: Effect of organic acid pretreatment

Cotton gin waste, a lignocellulosic biomass, is a potential raw material for bioethanol production. However, an appropriate pretreatment strategy is essential for the removal of lignin, thereby releasing cellulose and hemicellulose as fermentable sugar components from lignocellulosic biomass. Pretreatment using dilute sulfuric acid is the most efficient and widely used method to recover pentose sugars from lignocellulosic waste [70,122]. However, besides hazardous, this acid pretreatment produces toxic by-products such as furfural, 5 HMF, weak acids and phenol which affect the growth of yeast during fermentation leading to lower bioethanol yield [79]. A large amount of gypsum is also produced during sulfuric acid pretreatment which further affects the downstream processes [7,80]. In this context, pretreatment of biomass using organic acid might be attractive as it produces less toxic by-products and the method is environment-friendly [8,138]. However, sparse information is available on the use of organic acid for the pretreatment of lignocellulosic waste and to the best of our knowledge; no study is reported on the pretreatment of cotton gin waste using an organic acid so far.

Keeping these in view, this present study evaluates the efficiency of various organic acids such as oxalic acid, lactic acid, maleic acid and citric acid towards the pretreatment of cotton gin waste and the result was compared with the conventional dilute sulfuric acid pretreatment. The pretreated biomass was further evaluated for the release of sugar by enzymatic hydrolysis and subsequently bioethanol production by fermentation of

hydrolysates. Furthermore, the lignin degradation pattern of the pretreated cotton gin waste was assessed by FTIR, XRD and SEM study. The results and discussion of this research work are described in this chapter.

5.1.1 Composition analysis of cotton gin wastes

The sample collected from the cotton mill of Shree Ambica Agro Industries Ltd., Orissa, India is shown in figure 5.1. The composition of the cotton gin waste was analyzed and the analytical data is depicted in Table 4 and 5. Cotton gin waste was found to contain cellulose, hemicellulose and lignin as the major components. The biomass can be a potential feedstock for bioethanol production which is evident from its high carbohydrate content (55%).

Figure 5.1: Cotton gin waste collected from the cotton mill of Shree Ambica Agro Industries Ltd. India

The proximate analysis classifies the biomass in terms of its moisture, ash and fixed carbon content based on the ultimate analysis of elemental carbon (C), nitrogen (N), hydrogen (H) sulfur (S) and oxygen (O). The biomass elemental analysis result is important to evaluate the ratio between the main elements present in biomass, especially C/N ratio. A high C/N ratio implies that the material can be easily burnt and suitable for thermo-chemical conversion; on the contrary, a low C/N ratio indicates that the biomass is suitable for biochemical processes [72]. The C/N ratio of *cotton gin waste* was measured

to be 22.7 which indicate that the biomass can be easily converted to bioethanol through biotransformation.

Table 4: Composition of cotton gin waste

Biomass polymers (wt %)				**Sugar monomers (%)**		
Cellulose	Hemicellulose	Lignin	Ethanol extractives	Glucose	Xylose	Arabinose
40.3±0.01	15±0.01	19.8±0.30	9.21±0.32	47.01±0.37	18.5±0.74	5.58±0.84

Table 5: Proximate and ultimate analysis of cotton gin waste

Proximate analysis (wt %)				**Ultimate analysis (wt %)**				
Moisture	Ash	Other impurity	Fixed carbon	C	H	N	S	O
8.5±0.37	9±0.11	7.4±0.45	11.64±0.34	37.85±0.75	5.4±0.33	1.50±0.22	0.42±0.43	45.30±0.43

5.1.2 Evaluation of organic acid pretreatment of cotton gin waste

The efficiency of different organic acids namely citric, maleic, oxalic and lactic acid towards the pretreatment of cotton gin waste was evaluated in terms of the release of C5 sugar components and formation of inhibitors. The pretreatment results are shown in Table 7. As indicated, among the organic acids, maleic acid was found to be the most efficient as it provided the maximum C5 sugar recovery of 84% with 127.4±1.05 g/g sugar yield. The most of the sugar released was belong to xylose with 126.05±0.74 g/g yield and 12.60 g/l concentration. The pretreatment result was comparable to the pretreatment using sulfuric acid resulting 90 % C5 sugar recovery, 133.02±1.06 g/g sugar yield, 132.03±0.20 g/g xylose yield, 13.22 g/l xylose concentration at 150°C, 45min pretreatment time and 500mM acid concentration. A mild cellulose hydrolysis was also evident from the presence of glucose (1.74 g/l) in the liquid fraction of maleic acid treated sample which may be due to high temperature and high acid concentration [69]. The glucose concentration was slightly lower than dilute sulfuric acid and comparable to the oxalic acid pretreatment. However, sulfuric acid pretreatment produced a higher amount of toxic by-products including furfural (1.83±0.04 g/l), HMF (0.70±0.11 g/l), phenol (0.45±0.88 g/l) and acetic acid (3.02±0.05 g/l) followed by oxalic acid pretreatment. This represents the higher severity of inorganic acid in terms of producing inhibitory toxic compounds as also reported earlier [8,69]. The pH of different acid before treatment is

shown in Table 6. The calculated combined severity from the three factors such as temperature, pH and pretreatment time is shown in Table 7. The overall trend of toxic by-products follows dilute sulfuric > oxalic > maleic > citric > lactic acid. The resulting concentration of HMF and acetic acid produced by maleic, lactic and citric acid is not high enough to be inhibitory to yeasts used in bioethanol fermentation [139]. Except oxalic acid, other organic acids have also shown better performance in terms of solid recovery than sulfuric acid. All taken together, maleic acid is found to be the most effective pretreatment agent achieving the maximum xylose yield and producing inhibitory compounds within the permissible limits which may avoid the detoxification step involved in conventional inorganic acid treatment to reduce the inhibitory effect of toxic byproducts produced during pretreatment. Thus, maleic acid pretreated biomass was used for further study and dilute sulfuric acid pretreated biomass was taken for comparison.

Table 6: Initial pH values of the different acid

Acid type	**pH before treatment**
Sulfuric acid	0.28
Oxalic acid	0.70
Maleic acid	0.98
Citric acid	1.45
Lactic acid	1.65

Table 7: Release of xylose sugar, combined severity factor, solid recovery, furfural, HMF and acetic acid concentration of five dilute acids at 150°C, 45 min pretreatment time and 500mM acid concentration

Acid type	**CS**	**Xylose (g/g)**	**Solid recovery (%)**	**Phenol (g/l)**	**Furfural (g/l)**	**HMF (g/l)**	**Acetic acid (g/l)**
Sulfuric acid	2.84	130.53±0.20	65	0.45	1.83±0.04	0.70±0.11	3.02±0.05
Oxalic acid	2.42	125.92±0.11	70	0.15	1.30±0.03	0.41±0.13	2.26±0.23
Maleic acid	2.15	126.05±0.75	77	0.10	0.75±0.02	0.22±0.10	1.02±0.04
Citric acid	1.57	109.32±0.41	80	0.05	0.15±0.05	0.05±0.15	0.47±0.15
Lactic acid	1.74	100.42±0.52	82	0.03	0.14±0.07	0.04±0.18	0.36±0.42

5.1.3 Optimization of pretreatment parameters

The effect of key parameters such as temperature (100-150°C), time (30-60min) and acid concentration (300-700mM) on the pretreatment using maleic and sulfuric acid was evaluated to establish the most favorable pretreatment condition to maximize the release of C5 and C6 sugar components from cotton gin waste. The experimental results are shown in Table 8 and 9. The yield of sugar was increased with increase in temperature till 130°C at 500mM acid concentration and 45 min pretreatment time. A slight decline in sugar yield was observed with the further increase of these parameters (150°C, 60min and 700mM concentration) which may be due to degradation of sugars at severe process conditions [69]. Among the pretreatment agents, the maximum sugar release was obtained with maleic acid (122.45±0.67 g/g total C5 sugar yield with xylose concentration 12.15 g/l and yield 121.53±1.01 g/g). A comparable sugar release (128.24±1.20 total C5 sugar yield, 12.72 g/l xylose concentration and 127.26±1.17 g/g xylose yield) was also achieved by sulfuric acid at 150°C, 60min and 700mM. Hence, a minute difference in sugar concentration was observed when the substrate was treated with 500mM at 130°C for 45 min. The corresponding maleic and sulfuric acid pretreated hydrolysates obtained were (g/l): xylose (12.43±1.71 g/g and 13.11±0.70 g/l), glucose (1.06±0.50 and 1.24±0.49 g/l), arabinose (0.95±0.65 and 0.98±0.83 g/l). Furthermore, an enhanced amount of inhibitory compound was formed when pretreatment was carried out using dilute sulfuric acid at high temperature and prolonged pretreatment as also reported earlier [138]. The concentration of phenol and furfural has a more severe effect than HMF and acetic acid which are comparatively low in acid hydrolysate obtained from maleic acid treated biomass [76]. The corresponding phenol and furfural concentration were 0.07 g/l and 0.60 g/l which are lower than those formed with conventional acid pretreated biomass at the optimum condition as shown in Table 9. A relatively small amount of the glucan was also converted to monomeric glucose during the pretreatment as also discussed in the previous section.

Table 8: Effect of different process parameters (time, temperature and acid concentration) on the release of sugars, phenol and furfural during maleic acid pretreatment of cotton gin waste

Temp(^{0}C)	Acid conc.(mM)	30 min			45 min			60 min		
		Xylose(g/l)	Phenol(g/l)	Furfural(g/l)	xylose (g/l)	Phenol(g/l)	Furfural(g/l)	xylose (g/l)	Phenol(g/l)	Furfural(g/l)
100	**300**	3.87±0.88	0.02±0.04	0.10±0.32	5.44±1.16	0.02±0.33	0.16±0.20	5.91±1.30	0.05±0.43	0.22±0.11
	500	4.74±1.12	0.03±0.06	0.13±1.12	7.85±1.33	0.04±0.14	0.24±0.11	8.55±1.04	0.07 ±0.11	0.40±0.27
	700	5.51±1.33	0.05±0.11	0.15±0.55	8.94±1.41	0.05±0.40	0.35±0.34	9.21±0.76	0.08 ±0.41	0.55±0.24
130	**300**	6.48±1.40	0.03±0.12	0.18±0.43	10.82±1.70	0.04±0.52	0.53±0.44	11.28±1.15	0.08±0.63	0.72±0.33
	500	6.81±1.56	0.05±0.25	0.20±0.67	12.43±1.45	0.07±0.25	0.60±0.22	12.14±1.05	0.12±0.28	0.79±0.40
	700	7.02±1.11	0.06±0.44	0.22±0.72	12.49±1.30	0. 08±0.50	0.80±0.23	12.34±1.34	0.15±0.30	0.80±0.59
150	**300**	7.21±1.48	0.05±0.31	0.25±0.80	12.12±1.25	0.08±0.28	0.70±0.77	12.20±1.22	0.18±0.65	0.75±0.09
	500	7.73±1.76	0.06±0.17	0.27±0.19	12.45±1.38	0.10±0.37	0.78±0.08	11.99±1.06	0.20±0.71	0.90±0.11
	700	8.02±1.18	0.07±0.06	0.30±0.40	12.50±1.25	0.12±0.50	0.83±0.21	11.86±1.28	0.22±0.84	0.13±0.66

Table 9: Effect of different process parameters (time, temperature and concentration) on the release of sugars, phenol and furfural during sulfuric acid pretreatment of cotton gin waste

Temp(^{0}C)	Acid conc.(mM)	30 min			45 min			60 min		
		Xylose (g/l)	Phenol(g/l)	Furfural(g/l)	Xylose (g/l)	Phenol(g/l)	Furfural(g/l)	Xylose (g/l)	Phenol(g/l)	Furfural(g/l)
100	**300**	4.27±1.22	0.10±0.84	0.10±0.04	5.86±1.10	0.15±0.41	0.16±0.34	6.01±1.22	0.13±0.87	0.22±0.31
	500	5.34±1.53	0.13±1.01	0.15±0.06	8.39±1.41	0.19±0.13	0.24±0.25	8.63±1.34	0.28±0.13	0.40±0.44
	700	6.01±1.21	0.15±0.65	0.21±0.11	10.04±1.27	0.26±0.22	0.35±0.60	10.21±0.64	0.30±0.45	0.55±0.54
130	**300**	6.58±1.33	0.18±0.52	0.35±0.12	11.02±1.08	0.33±0.28	0.53±0.52	11.47±1.35	0.37±0.55	1.07±0.30
	500	7.01±1.05	0.20±0.72	0.41±0.25	12.85±1.12	0.45±0.17	1.15±0.40	12.86±0.67	0.42±0.26	1.35±0.26
	700	7.42±1.07	0.22±0.84	0.56±0.84	12.85±1.33	0.50±0.12	1.43±0.33	12.89±1.05	0.49±0.34	1.59±0.47
150	**300**	7.81±1.42	0.250.55	0.60±0.61	12.90±1.40	0.55±0.25	1.83±0.56	12.70±1.10	0.51±0.70	1.84±0.51
	500	8.03±1.22	0.270.62	0.66±0.17	13.32±1.06	0.59±0.40	2.14±0.70	13.01±0.88	0.55±0.65	2.47±0.46
	700	8.52±1.04	0.300.49	0.70±0.06	13.30±1.05	0.62±0.53	2.40±0.29	12.92±1.04	0.69±0.55	2.84±0.33

5.1.4 Detoxification of pretreated hydrolysate

As described in the earlier section that maleic acid pretreatment released toxic byproducts such as furfural, HMF and acetic acid is not enough to be inhibitory to yeasts in the fermentation process and hence the hydrolysate was not subjected to detoxification [7,8,76]. Therefore, sulfuric acid pretreated hydrolysate was detoxified using the methods of overliming and activated charcoal adsorption [69,70] that resulted in maximum removal of furfural (97.5%), HMF(96%) and phenol (98.7%) as depicted in Table 10. The percentage removal of various inhibitors using the only overliming was as follows: furfurals (42.1%), HMF (73.5%) and phenolics (28.2%), while activated charcoal resulted in the removal of 55.4% furfural, 22.5% HMF and 70.5% phenol. Furthermore, a small amount (8.5%) of sugar was reduced during detoxification process which was also reported earlier [70]. Therefore, as expected the sequential use of overliming and activated charcoal adsorption has been proved to be superior than using individual counterpart.

Table 10: Effect of detoxification on sugar content and removal of toxic by-products

Detoxification treatment	**Xylose (g/l)**	**Phenolics Removal (%)**	**Furfural removal (%)**	**HMF removal (%)**
None (undetoxified)	12.85±0.70	0	0	0
Overliming	12.2±0.50	28.2	42.1	73.5
Activated charcoal	12.6±0.33	70.5	55.4	22.5
Overliming+ activated charcoal	11.63±0.26	98.7	97.5	96

5.1.5 Delignification of acid pretreated biomass

During acid pretreatment, though a certain percentage of lignin is removed, most of the lignin remains intact to the cellulosic substrate. Hence, the removal of lignin from the biomass is an essential step that improves the crystalline structure of cellulose and facilitates substrate accessibility by hydrolytic enzymes [30]. Therefore, a suitable delignification method is important for the improvement of enzymatic hydrolysis of pretreated biomass in order to maximize sugar yield. In this context, the chemical delignification process is reported not only for delignification but it also acts as a swelling agent, which enhances the accessibility of enzyme in the surface area of biomass [69].

Therefore, pre-treated cotton gin waste was treated with a mixture of 5% (w/v) sodium sulphite and 3% (w/v) sodium chlorite solution as delignifying agents [69,70]. Figure 5.2 revealed 80% delignification at 30 min exposure time and 140°C. The maximum lignin removal of 88% was obtained with maleic acid pre-treated biomass which is also comparable to the lignin removal using sulfuric acid pretreated biomass (89%) achieved at an optimum condition of 140°C and 45 min as shown in figure 5.3. However, a higher phenolic concentration (17.54±0.88 g/l) was formed with sulfuric acid in comparison to maleic acid (17.35±0.72 g/l) shown in figure 8. Furthermore, the longer exposure time (60 min) was not favorable for delignification; where as the phenolic release was increased with the increase of temperature and time.

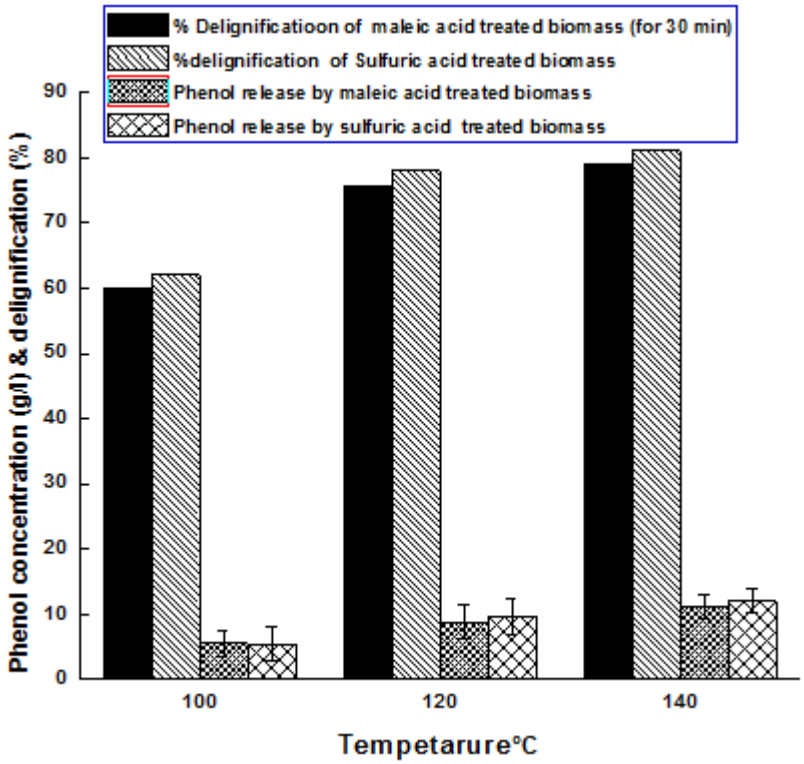


Figure 5.2: The release of phenol during the delignification of maleic acid and sulfuric acid pretreated biomass of cotton gin waste at different temperature (100, 120 and 140°C) for 30 min as exposure time

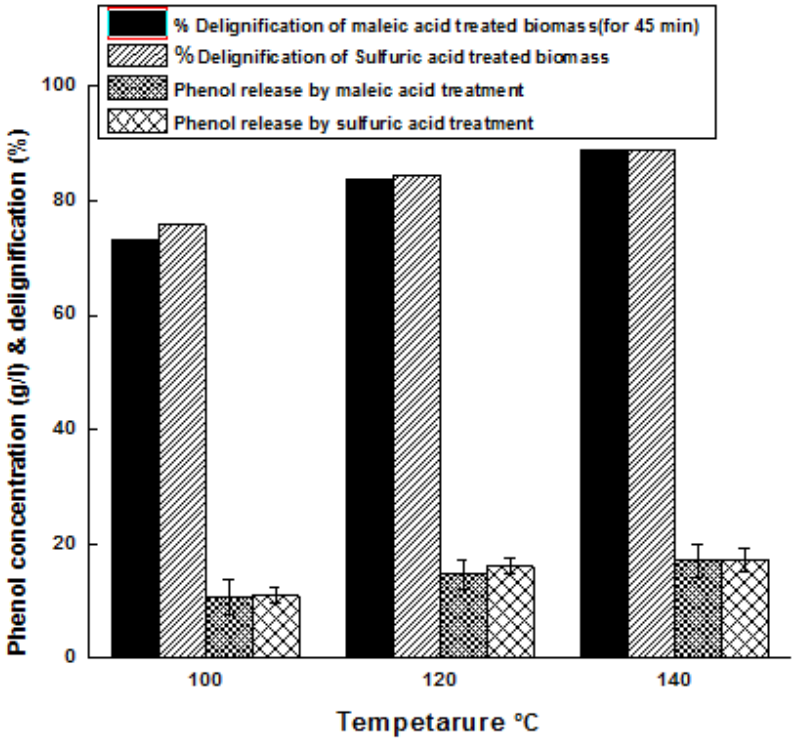


Figure 5.3: The release of phenol during the delignification of maleic acid and sulfuric acid pretreated cotton gin waste at different temperature (100, 120 and 140°C) for 30 min as exposure time

5.1.6 FTIR, XRD and SEM analysis of untreated and pretreated cotton gin waste

The FTIR study was performed to demonstrate the change in the functional group present in the raw biomass due to the removal of lignin and hemicellulose by breaking of bonds during pretreatment. A prominent band of hemicellulose observed at 1370 cm^{1} in untreated biomass was subsequently removed in acid treated substrate. The acid treated substrate showed a reduction in the band at 1238 cm^{-1} and 1738 cm^{-1} representing the hemicellulose-lignin linkage and C=O stretching due to carbohydrate linked with lignin, respectively. This can be explained by the change in the degree of intermolecular H-bonding between OH group of cellulose and water [132]. The observed band at 3245-3325 cm^{-1} was attributed to O-H stretching vibration of the hydroxyl group. The FTIR spectrum of delignified acid-pretreated substrate has revealed noticeable changes at the bands relating to aromatic ring vibration at 1508-1509 $cm^{-1}$and 1457-1458 cm^{-1}.The disappearance of these bands showed that lignin was largely removed in comparison to polysaccharides during the chlorite pretreatment [140]. The typical value of cellulose is obtained at frequencies: 1428 cm^{1}, 1372 cm^{1}, 1331 cm^{1}, 1281 cm^{1}, 1164 cm^{1}, 1056 cm^{1} and 897 cm^{1} [141] as shown in figure 5.4.

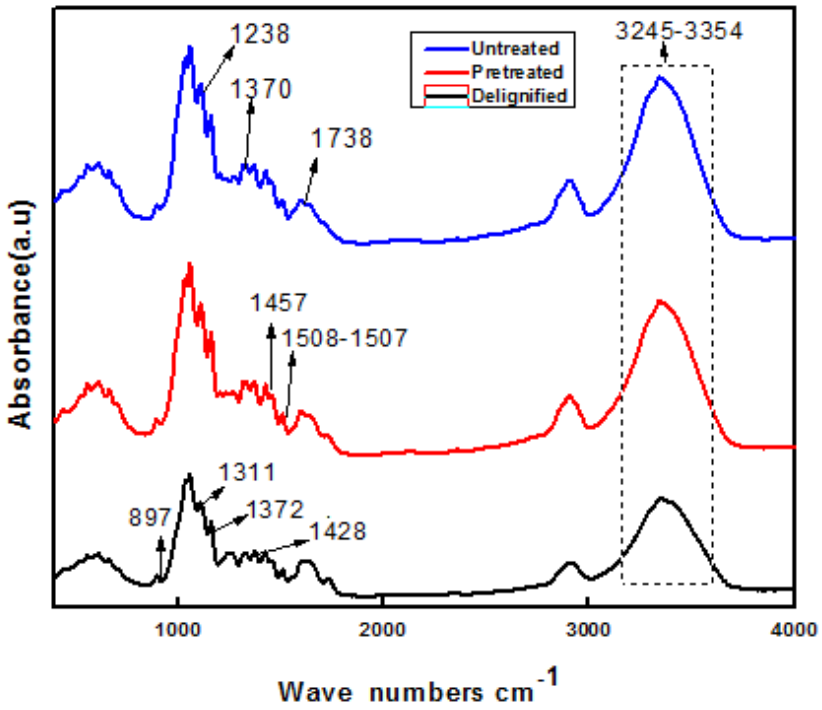


Figure 5.4: FTIR spectra of untreated, maleic acid pretreated and delignified cotton gin waste

Enzymatic hydrolysis is greatly influenced by the crystalline structure hence crystallinity of biomass. In lignocellulosic waste cellulose is the crystalline component whereas hemicelluloses and lignin are amourphus [86]. Further, 70% of the cellulose is in the crystalline region, therefore, the effect of the removal of lignin and hemicellulose on the crystallinity of the pretreated biomass by XRD shown in figure 5.5. The crystallinity of biomass is known to be a factor of influencing the enzymatic hydrolysis and raises the enzymatic digestibility [142]. XRD for untreated and pretreated samples exhibited similar crystalline patterns. The widths at half height for the peaks at 2θ =17º and 26º were similar for all samples except a high haziness in the untreated sample and more clarity in the peak of the pretreated sample. The cellulose crystallinity value of an untreated sample of cotton gin waste is 18.36% while that of the delignified sample is 30.54% representing an efficient improvement in crystallinity of the sample. The crystallinity of the pretreated sample was increased due to the removal of lignin and hemicellulose [132]. It is expected that amorphous region present in between the regular crystalline region is subjected to

attack and well exposed (crystalline region) by the removal of lignin and hemicellulose with improved crystallinity which was observed mainly after delignification.

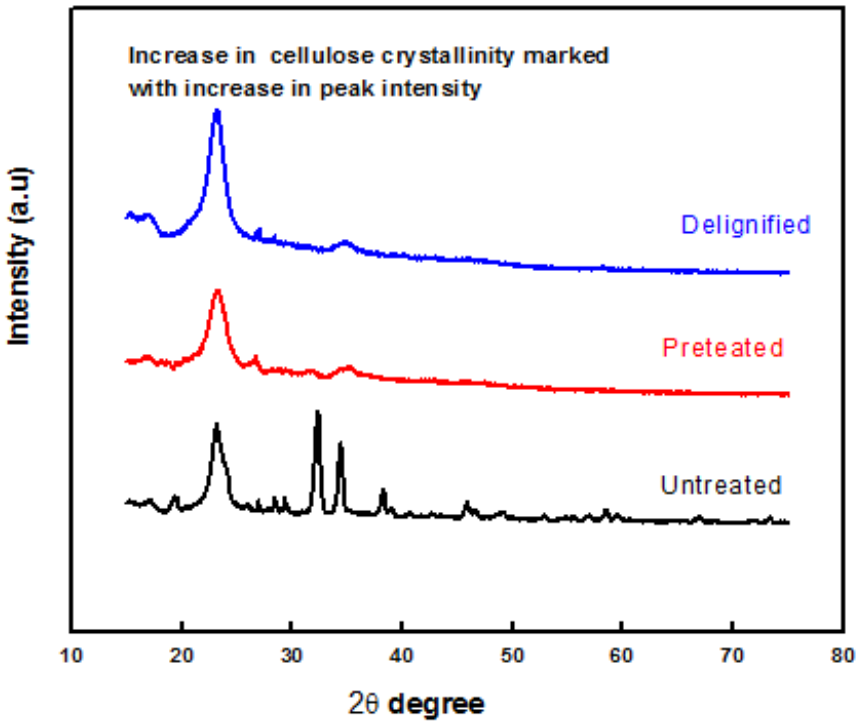


Figure 5.5: XRD analysis of untreated, maleic acid pretreated and delignified cotton gin waste

Scanning electron micrographs (SEM) images of (figure 5.6) untreated, pretreated and delignified cotton gin waste were analyzed to access any change in surface morphology that might have occurred during pretreatment. As indicated, untreated and pretreated biomass has shown variation in their micro-structure representing the variation in exposure of internal binding sites available to enzymatic hydrolysis. The untreated sample shows oriented fibers distributed over the whole region. Whereas, the pretreated sample shows partially degraded etched fibers indicating the influence of acid treatment on biomass. Furthermore, some of the macrofibrils remain separated and other was in agglomerated form. The significant change in the surface topography of the maleic pretreated biomass is due to the cleavage of the amorphous region of cellulose with retention of the crystalline fraction was observed. The pretreated sample shows some of the porous surface area which increased the better accessibility for further hydrolysid. The fibrous layers of delignified biomass show a heavy breakage on the surface area with defibrillation (free of trenches) at a large magnification which results in the breaking of

covalent bonds between lignin and cellulose. Hence, acid pretreatment and delignification of cotton gin waste improved enzyme accessibility for hydrolysis.

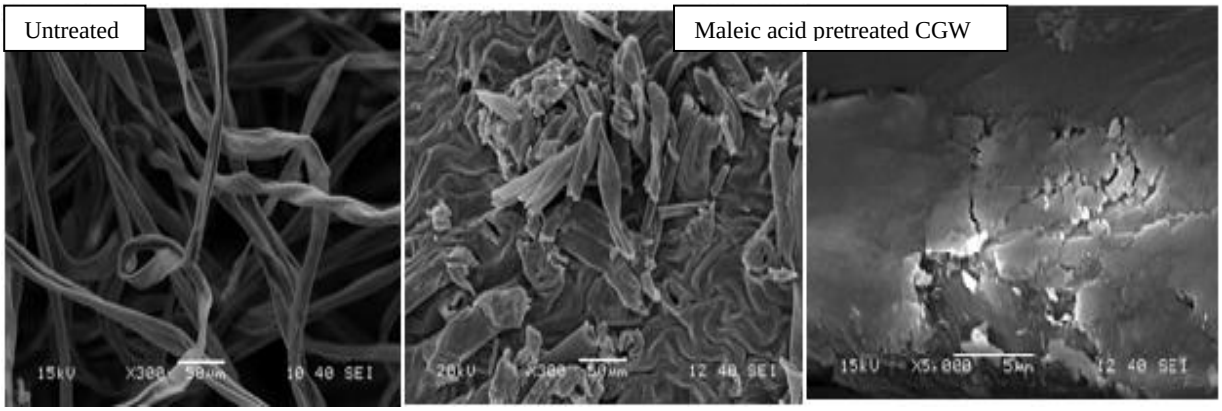


Figure 5.6: SEM analysis of untreated and maleic acid pretreated cotton gin waste

5.1.7 Enzymatic hydrolysis of delignified biomass

The major challenges in enzymatic hydrolysis lie with the reduction in cellulose crystallinity that hinders cellulose to be hydrolysed to release monomers sugar [70]. In this context, cellulase enzyme breaks the internal bonds of cellulose polymer to short chains and β-glucosidase reacts on the cello-oligosaccharides and cellobiose resulting the release of monomeric glucose units as fermentation components [70]. Acid pretreatment has a great influence on the efficient production of sugar by enzymatic hydrolysis [143]. As depicted in figure 5.7, the sugar concentration was increased with increase in hydrolysis time till 32h and thereafter the concentration becomes almost constant. The maximum 686.13 g/g saccharification yield was achieved with maleic acid pretreated biomass which was slightly higher than the sulfuric acid (675.26 g/g) pretreated waste (figure 5.8). The sugar concentration of 27.43±0.89 g/l and 27.05±1.02 g/l was obtained with maleic and sulfuric acid pretreated biomass. The corresponding rate of hydrolysis was measured to be 21.5 g/l/h and 21.1 g/l/h with maleic and sulfuric acid pretreated sample after 32h of hydrolysis. Overall 68% (w/w) saccharification was achieved with maleic acid treated biomass which is comparable to the sulfuric (67%) acid pretreated sample. Furthermore, sulfuric acid pretreatment of cotton gin waste achieved higher xylose release in comparison to organic acids but low C6 sugar released during enzymatic hydrolysis which was also reported earlier [71]. Earlier, Fockink reported maximum 62.1% glucose recovery on enzymatic hydrolysis by alkali pretreatment of cotton gin waste [144].

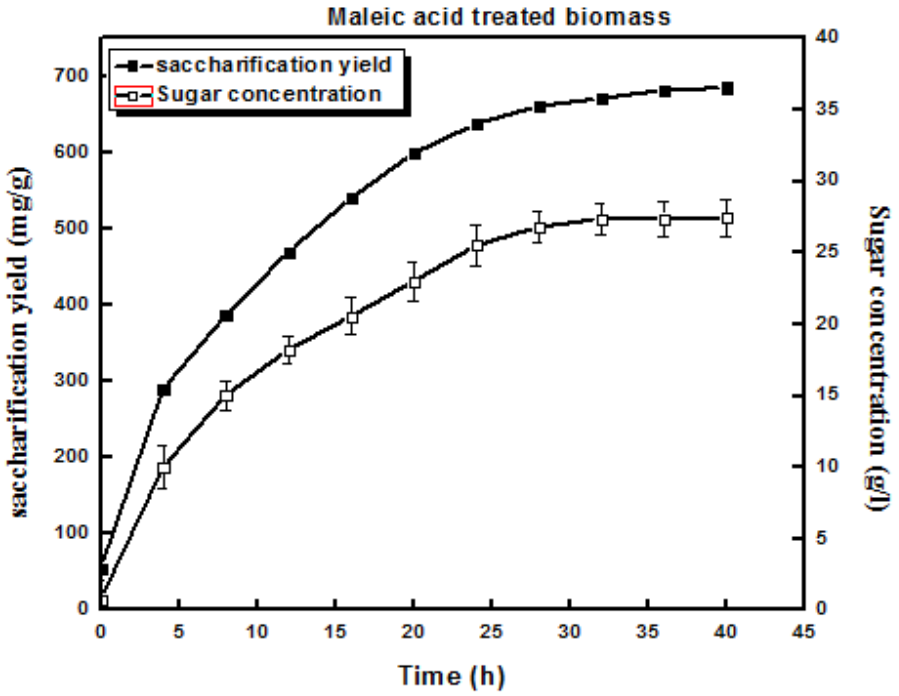


Figure 5.7: Enzymatic saccharification of maleic acid pretreated and delignified cotton gin waste at 50^0C, pH 5 and 150rpm

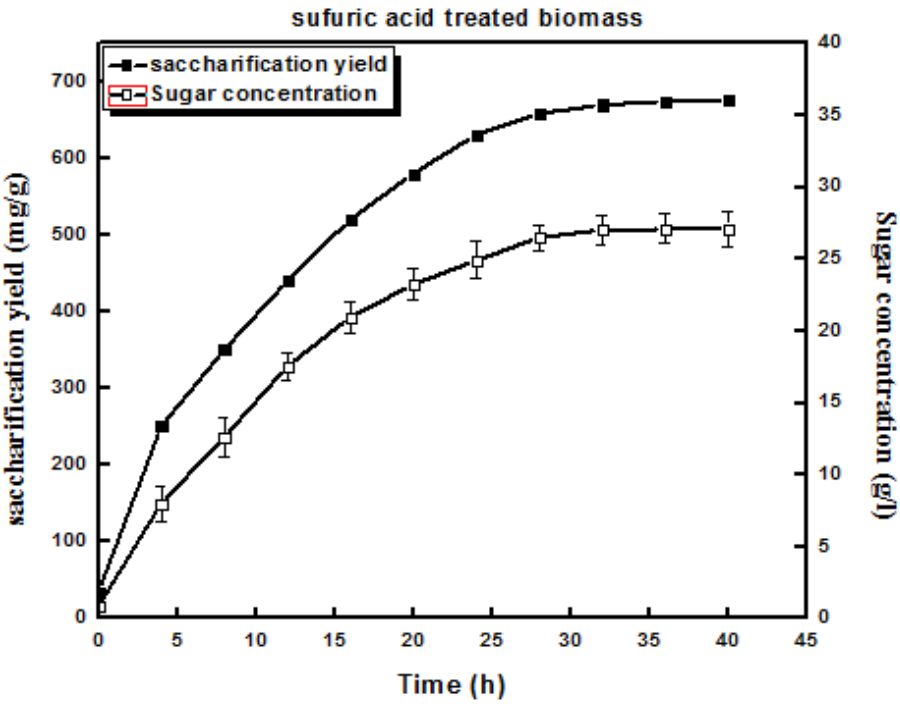


Figure 5.8: Enzymatic saccharification of sulfuric acid treated delignified cotton gin waste at 50^0C, pH 5 and 150rpm

5.1.8 Fermentation of acid and enzymatic hydrolysates to bioethanol

Fermentation is the final step of the conversion of cotton gin waste to produce bioethanol. Evaluation of bioethanol production by fermentation is of utmost importance to quantify the performance of the final process. The fermentation of the mixture of acid and enzymatic hydrolysates (41.75 g/l) obtained from maleic acid pretreated biomass was fermented with *S. cerevisiae* and *P. stipitis* which are considered to be the most efficient xylose and glucose fermenting yeast strains respectively [69,145]. Furthermore, the efficiency of fermentation by the mixture of acid and enzymatic hydrolysates using an individual, co-culture and sequential use of *S. cerevisiae* and *P. stipitis* yeast strains at 150 rpm and 30°C was evaluated. The fermentation profile is depicted in table 11. Among the three conditions, the sequential use of yeast strains achieved maximum (18.25±0.89 g/l) ethanol concentration (figure 5.9) with 86% theoretical yield, 90.7% sugar consumption and 2.04 g/l/h ethanol productivity than individual and co-culture. The results are in good agreement with the study of Li et al. [124], reporting 85% theoretical yield of ethanol using sequential use of *S. cerevisiae* and *P. stipitis* yeast strains.

Table 11: Fermentation of acid and enzymatic hydrolysates using co-culture, individual and sequential use of *S. cerevisiae* and *P. stipitis* at 150 rpm and 30°C

Yeast(s)	Max. sugar consumption %	Maximum ethanol conc.(g/l)	Theoretical yield of ethanol	Time of fermentation(h)
Co-culture	84%	17.05±1.03	80%	48
P. stipitis	78%	15.31±0.78	76%	64
S. cerevisiae	64%	11.04±1.08	62%	56
Sequentially	90.7%	18.25±0.89	86%	56

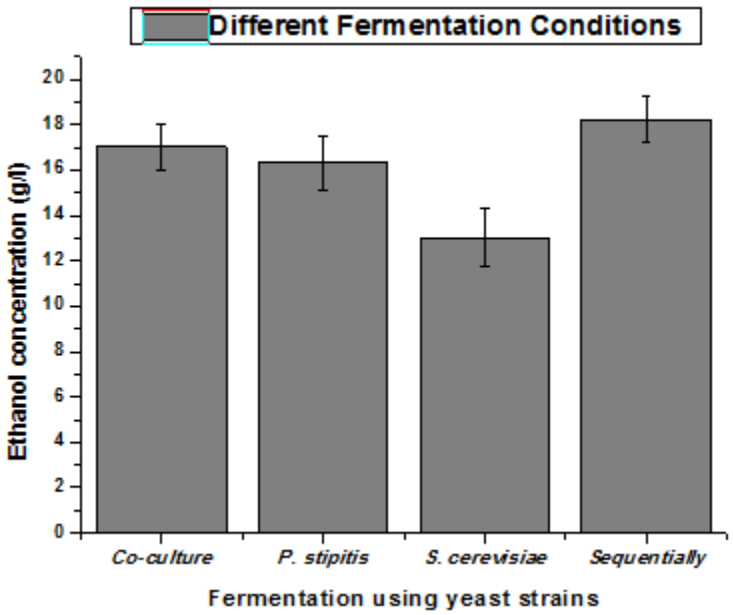


Figure 5.9: The maximum performance in terms of bioethanol concentration using yeast strains in different fermentation conditions

5.1.9 Influence of key parameters on bioethanol fermentation

The effort has been given to investigate the influence of the important fermentation parameters to maximize the bioethanol production. The mixture of acid and enzymatic hydrolysates (41.75 g/l) were fermented with sequential use of *S. cerevisiae* and *P. stipitis* at varying conditions such as agitation (150, 200, 250 & 300 rpm), temperature (25, 30, 35 & 40°C) and pH (4.5, 5, 5.5 & 6) for 64h to establish the optimum fermentation conditions which will facilitate the bioethanol fermentation.

Effect of agitation

Agitation in fermentation broth maintains the uniform mixing (homogeneous) of media in the bioreactor and adequate oxygen transfer [146]. Agitation speed is one of the important parameters which influence the bioethanol production and yeast growth in a bioreactor. The effect of agitation on bioethanol production is important for the successful progress of the fermentation. The effect of agitation speed on bioethanol production was, therefore, evaluated in the range 150-350rpm. The bioethanol concentration was increasing with increase in agitation speed and fermentation time till 48h as shown in figure 5.10. A decline in bioethanol concentration was observed when agitation speed was above 200rpm and 56h fermentation time. The maximum ethanol concentration of 17.8 ±1.04 g/l, 1.7 g/l/h productivity and 0.42 g/g ethanol yield were achieved at 200rpm, pH 5 and 30°C.

The results indicate that the agitation speed of 200 rpm was the most suitable for ethanol production by sequential use of yeast strains.

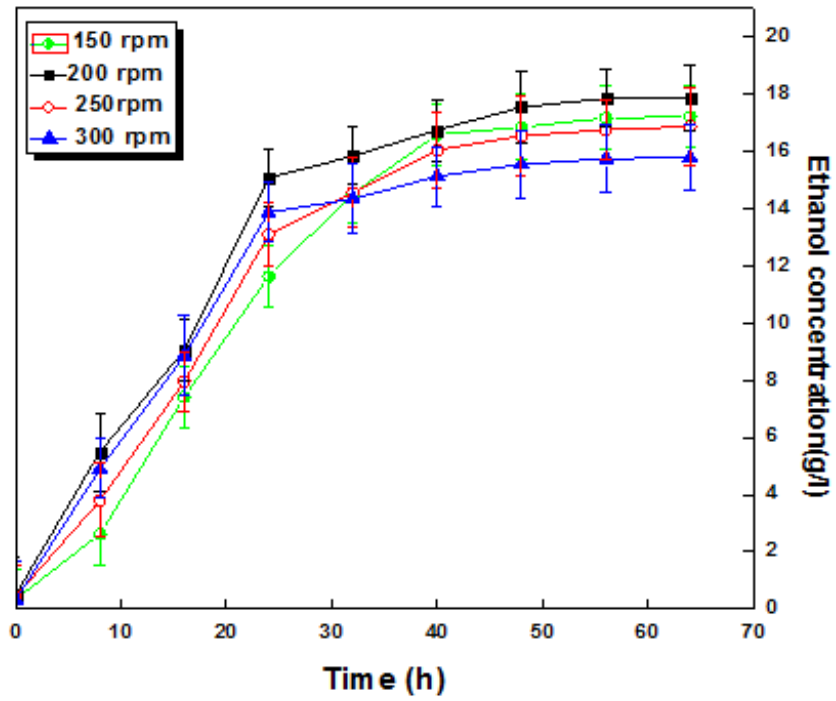


Figure 5.10: Influence of agitation speed on bioethanol concentration during 64h fermentation using 41.75 g/l total C5 and C6 sugar at constant pH 5 and 30°C

Effect of temperature

The temperature is reported to have a great influence on bioethanol fermentation. Generally, the temperature range between 30-40°C is favorable for yeast growth in fermentation medium [147]. Therefore, the fermentation experiment was carried out at varying temperature in the range of 25-40°C at constant pH 5 and 200 rpm. The results on the effect of temperature on ethanol concentration and yield are shown in figure 5.11. As it is observed, the rate of ethanol concentration was increased with increase in temperature from 25 °C to 33°C and then there is a decrease bioethanol production was observed at a higher temperature, though a comparable ethanol production was observed at 35°C. Therefore, 30°C was found to be the most favorable temperature with respect to higher ethanol concentration and rate of reaction achieving 18.2±1.26 g/l as maximum ethanol concentration, 1.93 g/l/h ethanol productivity and 0.44 g/g ethanol yield. The decrease in ethanol production at a higher temperature (40°C) is due to the deactivation of enzyme

present in yeast strains. A loss of enzyme activity and hence lower ethanol production at high temperature was also reported earlier [147].

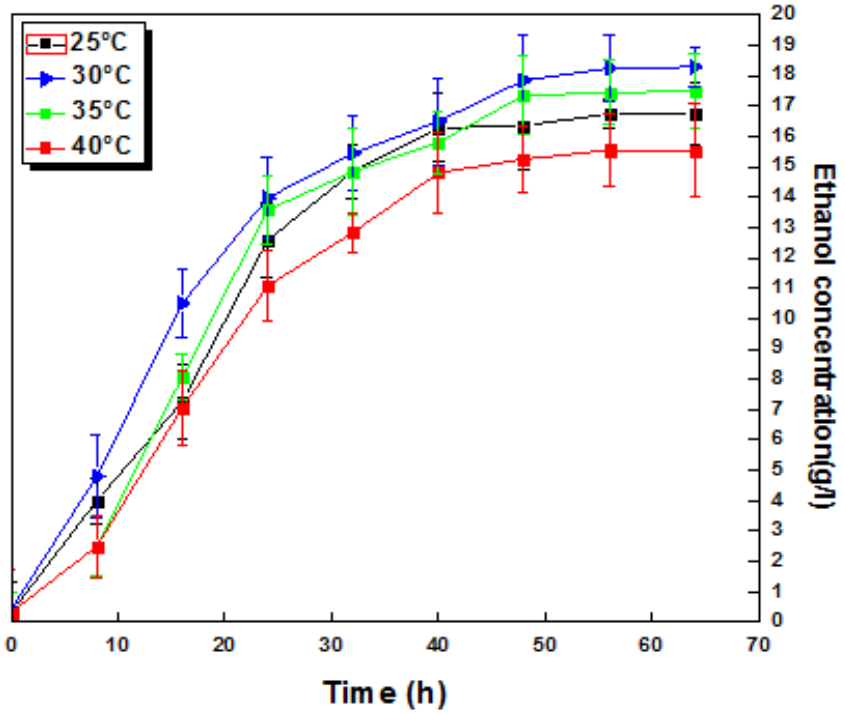


Figure 5.11: Influence of temperature on bioethanol concentration during 64h fermentation using 41.75 g/l total C5 and C6 sugar at constant pH 5 and 200rpm

Effect of pH

pH has a significance influence on yeast growth which ultimately affects bioethanol fermentation [147]. Therefore, the effect of pH was studied at varying range of pH 4.5-6 at a constant temperature of 30°C and 200 rpm agitation speed. The experimental results are depicted in figure 5.12. As indicated, the ethanol concentration was increased steadily till 56h at all pH though the rate of concentration varied considerably. However, the maximum ethanol concentration of 18.74±1.55 g/l was achieved at pH 5.5 followed by pH 5.0 (18.4 g/l). The lower activity of the yeast strain at pH 6 is because the pH is too low to activate the enzymes to react. Furthermore, maximum ethanol yield of 0.48 g/g, 0.30 g/g biomass yield and 2.25 g/l/h ethanol productivity were obtained with pH 5.5 is higher than the yields obtained at pH 4.5 and 6. The results suggested that pH 5.5 is the most favorable pH for bioethanol fermentation using sequential use of yeast strains. The

corresponding biomass growth and sugar consumption with respect to bioethanol production were shown in figure 18.

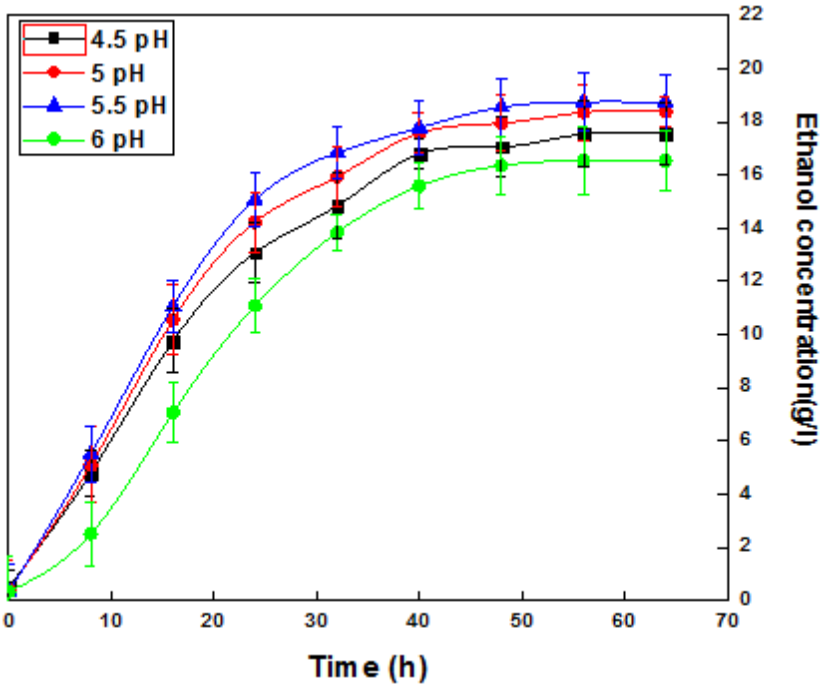


Figure 5.12: Influence of pH on bioethanol concentration during 64h fermentation using 41.75 g/l total C5 and C6 sugar at 30°C and 200rpm

Overall, from the above study, 30°C, pH 5.5 and 200 rpm were found to be most favorable fermentation conditions achieving 0.48 g/g ethanol yield, 2.25 g/l/h ethanol productivity, 18.74±1.06 g/l ethanol concentration and 88% theoretical yield. The bioethanol production was also compared with the dilute sulfuric acid pretreated CGW as shown in figure 5.13 and 5.14. As it is observed, slightly lower ethanol productivity (2.11 g/l/h), ethanol yield (0.45 g/g) and biomass yield (0.29 g/g) with 18.92±1.46 g/l concentration was obtained by fermentation of sulfuric acid pretreated hydrolysates. The efficiency of the fermentability of the detoxified and undetoxified sulfuric acid treated hydrolysates showed a marginal decrease (12%) in ethanol concentration with the undetoxified sample as depicted in figure 5.15. A mild 2% variation in bioethanol concentration was observed by maleic acid pretreated CGW.

The bioethanol production achieved using maleic acid pretreatment was quite higher than the previously reported value (7.1 g/l ethanol concentration and 0.4 g/l/h productivity) achieved by fermentation of hydrolysates derived from cotton gin waste using a hybrid

strain developed from *S. cerevisiae* and *P. stipitis* yeast strains [5]. In another study 0.31 g/g of ethanol yield using *S. cerevisiae* was reported using from cotton gin waste [16]. Even our results are better than the maximum reported value of 83% theoretical yield of ethanol from cotton gin waste using *E.coli* KO11 [38]. The fermentation efficiency is also favorable than the maximum 85% theoretical yield using sequential use of *S. cerevisiae* and *P. stipitis* yeast strains from rice straw biomass reported earlier [124].

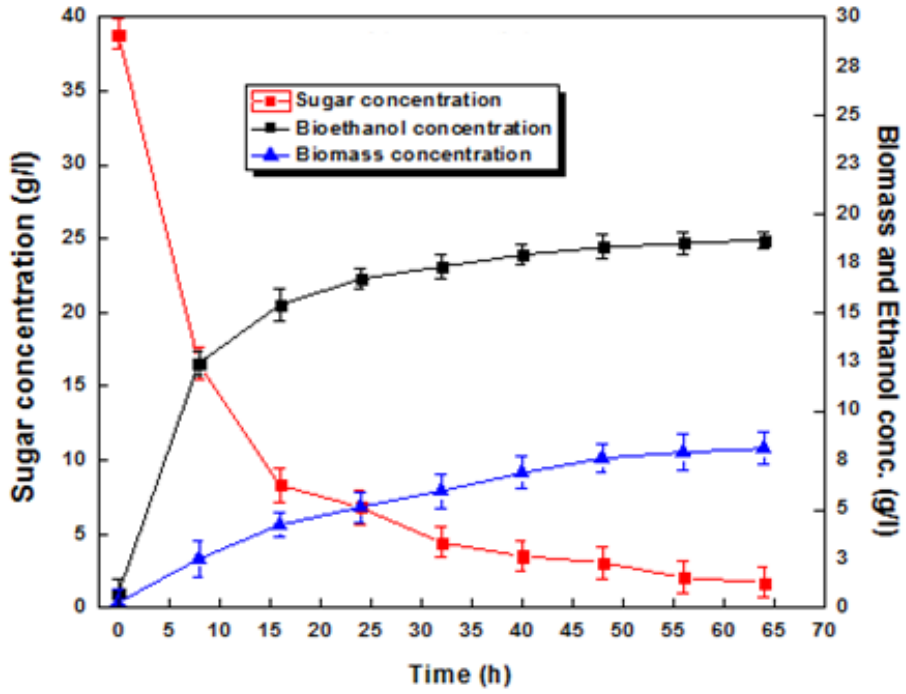


Figure 5.13: Fermentation of hydrolysate derived from maleic acid pretreated CGW using *S. cerevisiae* and *P. stipitis* yeast strains sequentially at optimum fermentation condition (30°C, pH 5.5 and 200 rpm)

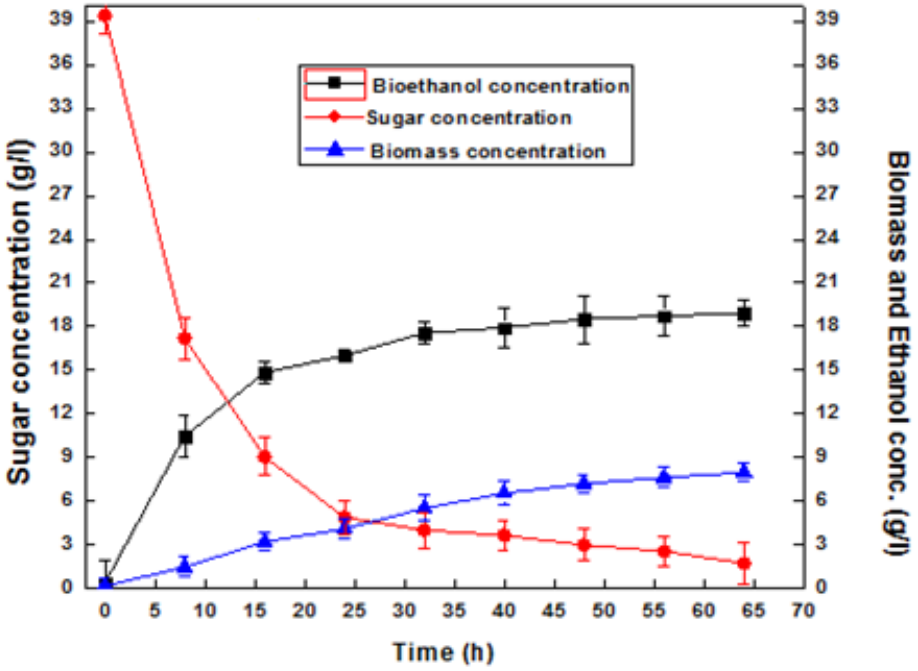


Figure 5.14: Fermentation of hydrolysate derived from sulfuric acid pretreated CGW using *S. cerevisiae* and *P. stipitis* yeast strains sequentially at optimum fermentation condition (30°C, pH 5.5 and 200 rpm)

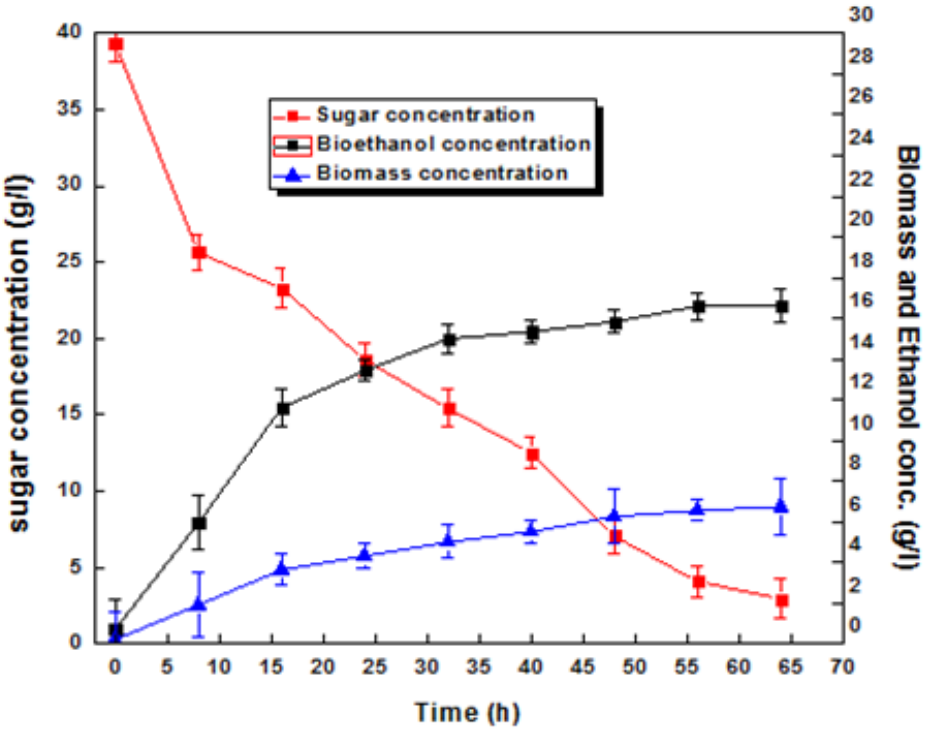


Figure 5.15: Fermentation of hydrolysate derived from sulfuric acid pretreated CGW (un-detoxified) using *S. cerevisiae* and *P. stipitis* yeast strains sequentially at optimum fermentation condition (30°C, pH 5.5 and 200 rpm)

5.2 Bioethanol production from cotton gin waste: Effect of fungal pretreatment

As it has been mentioned in the previous chapter that pretreatment of cotton gin waste is a prerequisite conversion step for bioethanol production. The pretreatment using dilute sulfuric acid, the most widely accepted method suffers from several drawbacks such as hazardous, high energy intensive, and formation of inhibitory toxic- byproducts [69,86]. Therefore, exploring an alternative pretreatment method is inevitable for the production of bioethanol from lignocellulosic biomass like cotton gin waste.

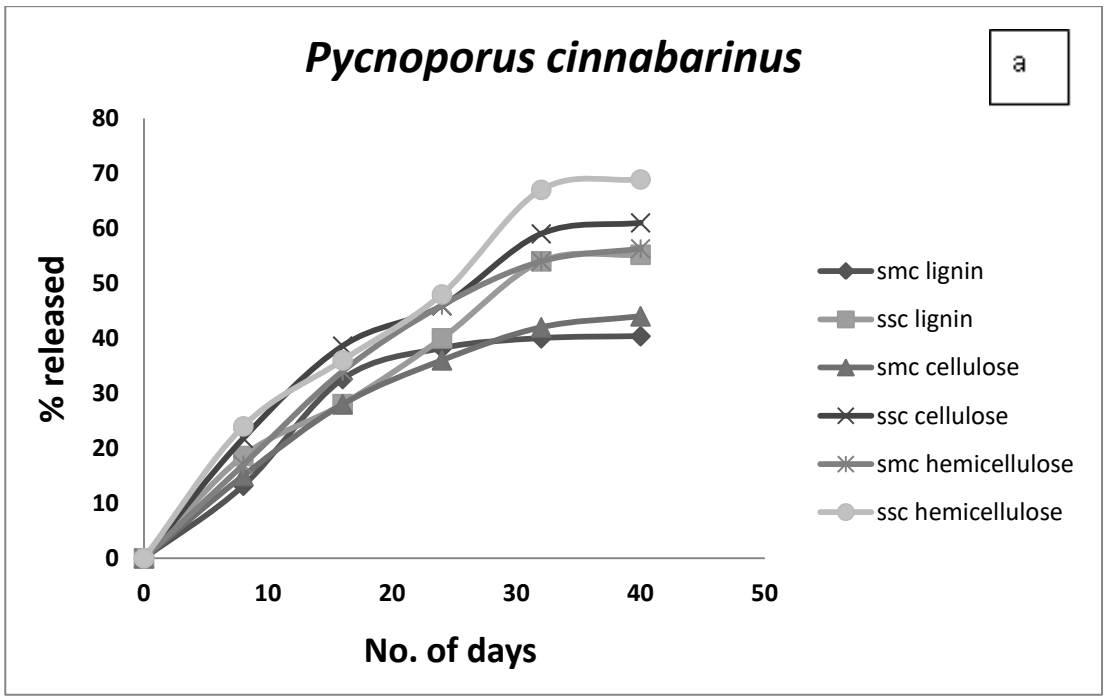
It is evident from the literature that biological pretreatment is advantageous over chemical pretreatment methods because of the requirement of mild reaction conditions, low energy and formation of minimal toxic byproduct [1]. Further, some fungal species belonging to white rot fungi has been reported to be the efficient microbial strains for biological pretreatment offering high and rapid lignin degradation than the other known organisms [149,150]. However, the pretreatment of cotton gin waste using fungal strains has been barely studied to produce bioethanol.

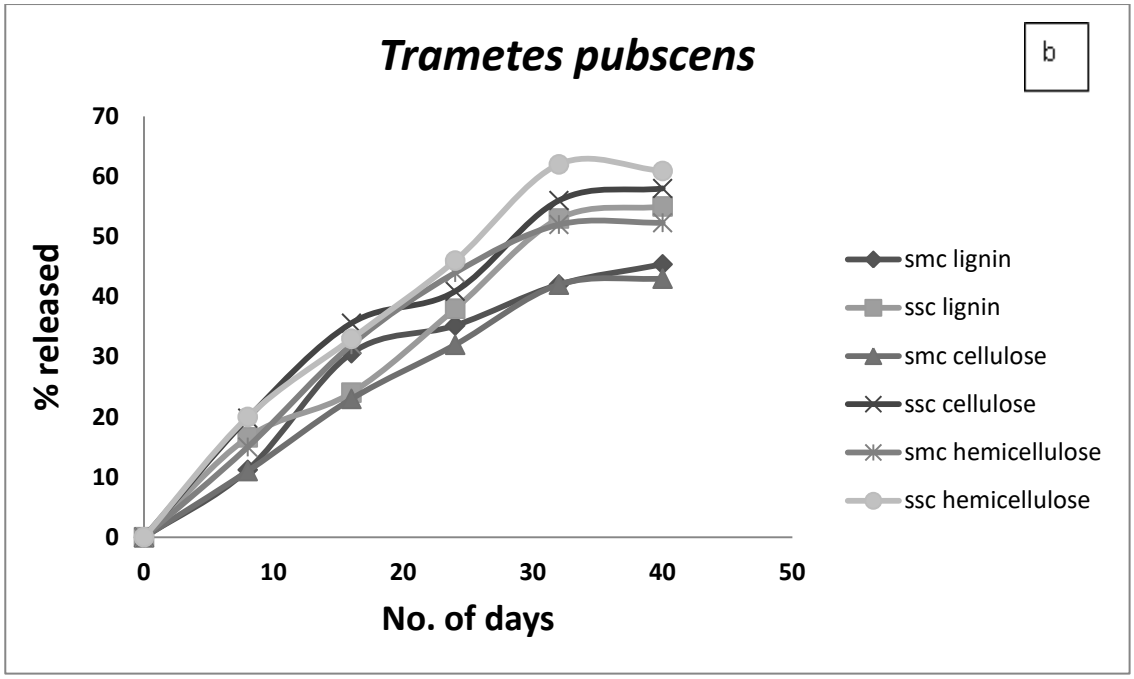
Keeping this in view, in this part of research work, the efficiency of some white rot fungi was evaluated for the pretreatment of cotton gin waste. The pretreated biomass obtained from the most efficient fungi was further hydrolysed to produce fermentable sugars which were subsequently converted to bioethanol as end products by fermentation. An effort was also given to optimizing the key pretreatment parameters by response surface model (RSM) based on central composite design (CCD).The pattern of lignin biodegradation was also examined through the analysis of SEM, FTIR and XRD. A result and discussion on this research work have been described in detail in this chapter.

5.2.1 Fungal pretreatment of cotton gin waste

The removal of lignin from the biomass by pretreatment process exposes the crystalline structure of cellulose and improves solubilization by water thereby facilities substrate accessibility by hydrolytic enzymes [30]. Therefore, delignification of cotton gin waste using four fungal strains such as *Trametes pubscens, Pycnoporus cinnabarinus, Phanerochaete chrysosporium* and *Pleurotus ostreatus* was carried out in solid and

submerge state (SSC and SMC) of cultivation and the experimental results are shown in figure 20. The maximum lignin removal of 55.2 and 40.2% was achieved by SSC and SMC using *Pycnoporus cinnabarinus*. The corresponding cellulose and hemicellulose release were calculated as 61.9 and 70% respectively in solid state and 44 and 56.3% in SMC shown in figure 5.16(a). Similarly, for *Trametes pubscens, Phanerochaete chrysosporium* and *Pleurotus ostreatus,* the lignin removal in solid state and submerge state culture were measured as 55, 40, 53.2 and 39, 52 and 38% respectively. *Pleurotus ostreatus* also showed a mild C6 sugar conversion during pretreatment. A substantial amount of lignin removal has been reported with *Trametes pubscens* releasing 61% cellulose and 68.9 % hemicelllulose in solid state cultivation (figure 5.16 b). In this study solid state cultivation using *Pycnoporus cinnabarinus* has shown the highest pretreatment efficiency than submerged state cultivation over other three fungi and this strain was used for further study. Furthermore, pretreatment outcome was superior using SSC than SMC.

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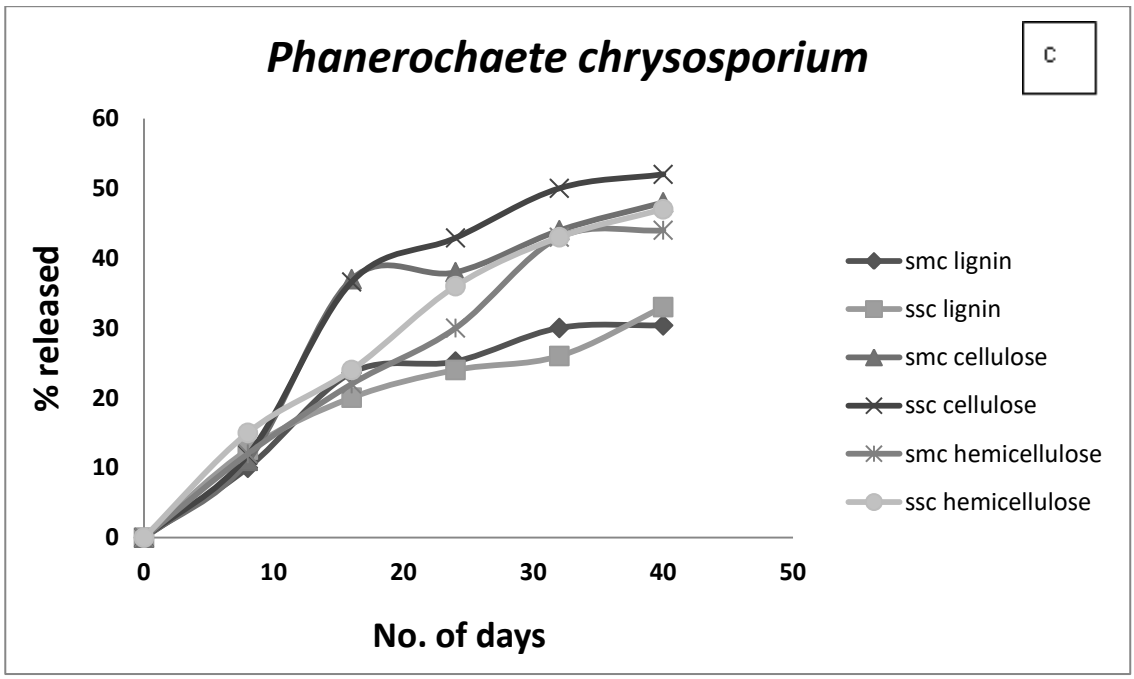
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smc hemicellulose
ssc hemicellulose

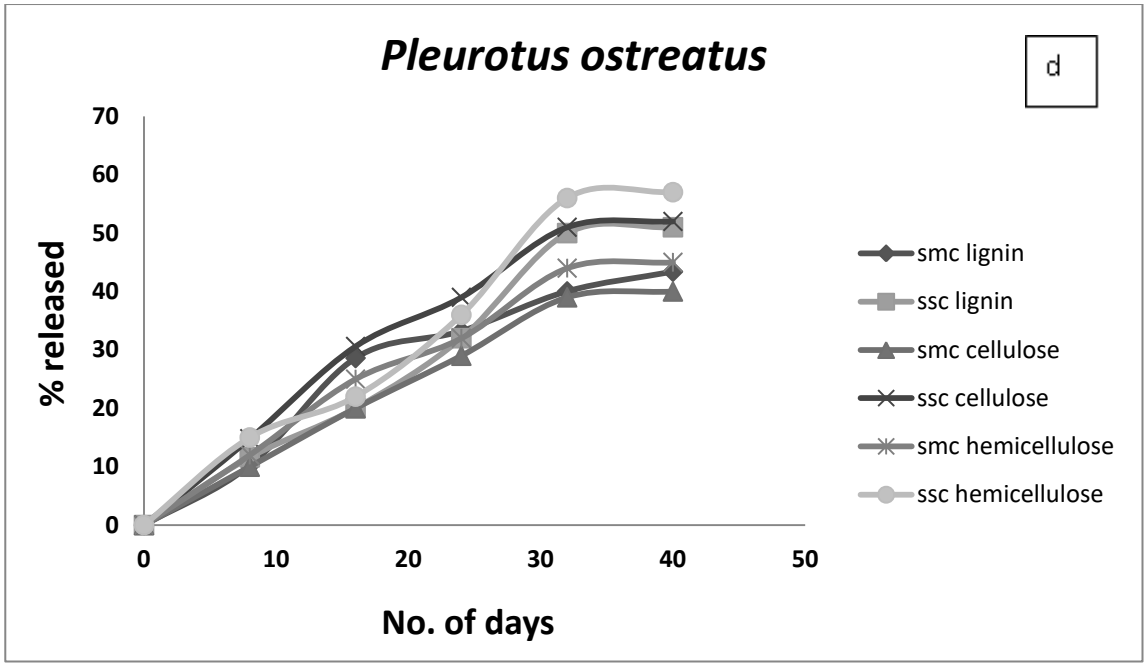


Figure 5.16: Effect of pretreatment on release of cellulose, hemicellulose and delignification by (a) *Pycnoporus cinnabarinus*, (b) *Trametes pubscens*, (c) *Phanerochaete chrysosporium* and (d) *Pleurotus ostreatus* in solid and submerge state of cultivation at 35°C, pH 4.5 and 100 rpm)

5.2.2 Effect of wash and heat-wash as pre-hydrolysis treatments

The effect of washing and heating of pretreated CGW on the lignin removal was investigated in this study. Besides lignin degradation, washing and heating removed fungal biomass by deactivating fungal activity on biomass, thereby increased enzyme accessibility to cellulose in pretreated samples is expected [151,152]. A marginal lignin removal of 0.4% and 0.3% was achieved by solid and submerge cultivation respectively. The corresponding cellulose and hemicellulose release were obtained measured as 0.2% and 0.4% in SSC, whereas their values in SMC were 0.3% and 0.2% respectively.

5.2.3 Optimization of pretreatment parameters

The optimization of key pretreatment parameters offers an improvement in economic feasibility of the process. Therefore, the optimization study on the three individual important parameters such as, pH (4 - 5), temperature (30 - 40°C) and rpm (100 -140) has been carried out to obtain the maximum % lignin degradation of cotton gin waste by RSM method using central composite design as shown in Table 12.

Table 12: Experimental design for the pretreatment of cotton gin waste using *Pycnoporus cinnabarinus* white wring fungal strain and its effect on delignification

Std Order	pH	Temp°C	RPM	% Delignification
1	4	30	100	52.9
2	5	30	100	53.0
3	4.	40	100	54.7
4	5	40	100	53.0
5	4	30	140	54.0
6	5	30	140	53.0
7	4	40	140	53.6
8	5	40	140	54.0
9	4	35	120	53.4
10	5	35	120	54.2
11	4	30	120	58.3
12	4	40	120	53.4
13	4	35	100	57.3
14	4	35	140	56.9
15	4.5	35	120	60.3
16	4.5	35	120	60.2
17	4.5	35	120	60.5
18	4.5	35	120.	60.0
19	4.5	35	120	60.0
20	4.5	35	120	59.6

As indicated, the percentage of delignification was obtained in the range of 52.9 to 60.5%. The mathematical expression relating % lignin degradation to the different independent variables and their interaction is expressed below in terms of coded factor:

$$Y = 59.38+0.24A_1-0.72A_2+0.56A_3-3.50A_1^2-0.80A_2^2-0.40A_3^2-0.86A_1A_2-0.81A_1A_3-0.28A_2A_3$$

Where, A_1, A_2 and A_3 represent pH, temperature and rpm and Y represents the experimental response.The individual action of all the three parameters studied, quadratic and interaction effects between the dependent variables were found to be significant from the regression model. Analysis of Variance (ANOVA) of the quadratic regression for biological pretreatment of cotton gin waste has been summarized in Table 13. The regression terms of the model have been shown an F-value of 12.67 and a very low probability value (< 0.001) which represents the significance of the model [66].

Table 13: ANOVA analysis of RSM model for biologically pretreated cotton gin waste

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Regression	9	114.102	114.1025	12.6781	12.87	0
Linear	3	8.896	8.896	2.9653	3.01	0.081
Square	3	93.313	93.3127	31.1042	31.58	0
Interaction	3	11.894	11.8938	3.9646	4.03	0.041
Residual Error	10	9.85	9.8495	0.985		
Lack-of-Fit	5	9.336	9.3362	1.8672	18.19	0.003
Pure Error	5	0.513	0.5133	0.1027		
Total	19	123.952				
R^2 = 0.9205						

DF= degree of freedom, SS= sum of squares, MS= mean sum of squares, F= Fisher's F value and P= probability

The square and interaction effects between the variables were found to be statistically significant with a P-value 0 and 0.04 respectively. The square term of the model was more significant than other effects with a higher F-value (31.58). The quality of the model was evaluated by the coefficient R^2 and its statistical significant was determined by F-test. For pretreated sample the R^2 values obtained as 0.9205 and hence justify the robustness of the model. Figure 5.17 shows the interaction of temperature and rpm at a constant pH, where as figure 5.18 shows the significance interaction between pH and rpm at constant temperature on lignin degradation of the sample. The three-dimensional plots show that the temperature at 35°C and shaking speed at 120 rpm caused an increase in the lignin degradation (%), yielding a maximum lignin degradation value of 60.5% after 32 days of solid-state cultivation. However, at a constant temperature, the interaction between shaking speed and pH gives the maximum value of delignification. In figure 5.19 the optimization has been performed with a variation in temperature and pH at constant rpm. From the experimental data, the above statistical model suggests the optimum predicted

the condition of pH, shaking speed and temperature 4.5, 138 rpm and 32°C respectively, which resulted in a high percentage of delignification. In order to check the reliability of predicted response, the experiment in triplicate has been performed under optimum predicted conditions. From these experiments, maximum delignification was found to be 61.2 % which is in good agreement with the predicted. From this design of experiments, the maximum release of cellulose and hemicellulose were found to be 62.01% and 70.04%.

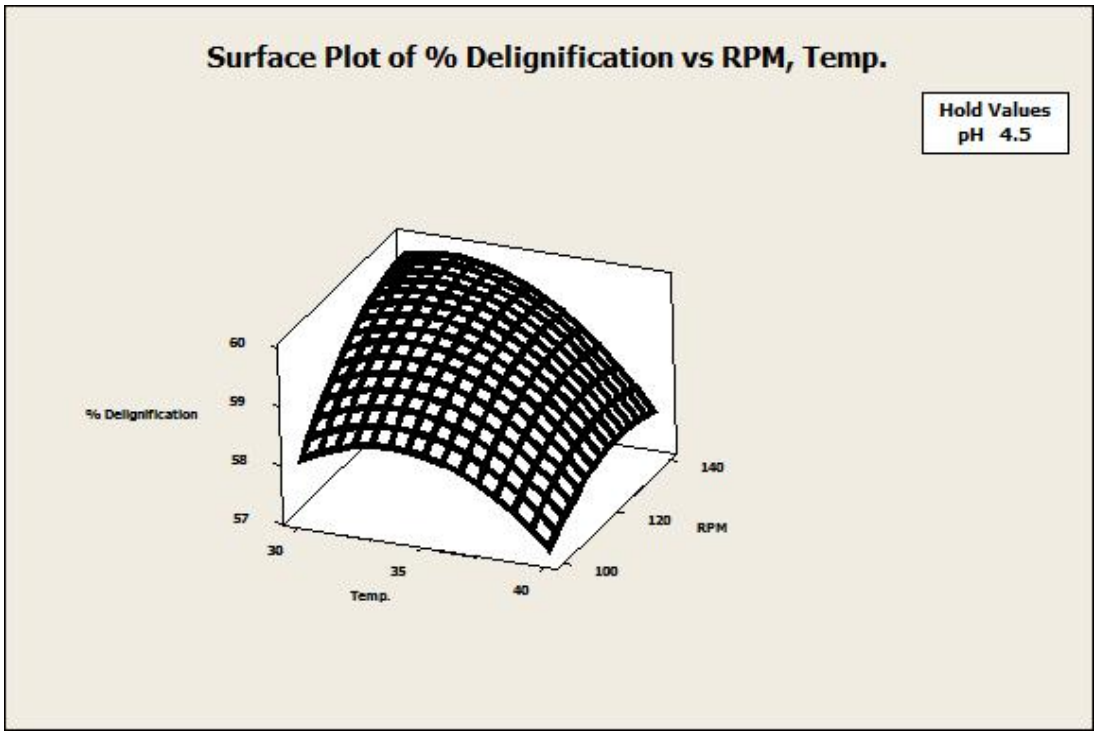


Figure 5.17: Response surface plots showing the effect of temperature and shaking speed on the pretreatment of cotton gin waste

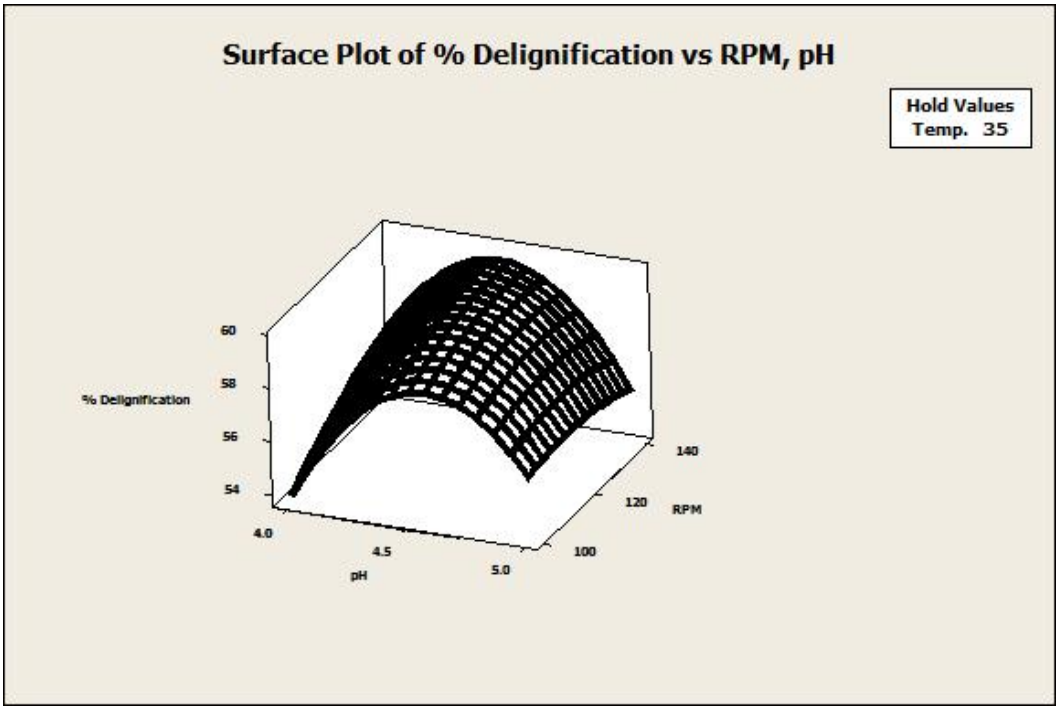


Figure 5.18: Response surface plots showing the effect of pH and shaking speed on the pretreatment of cotton gin waste

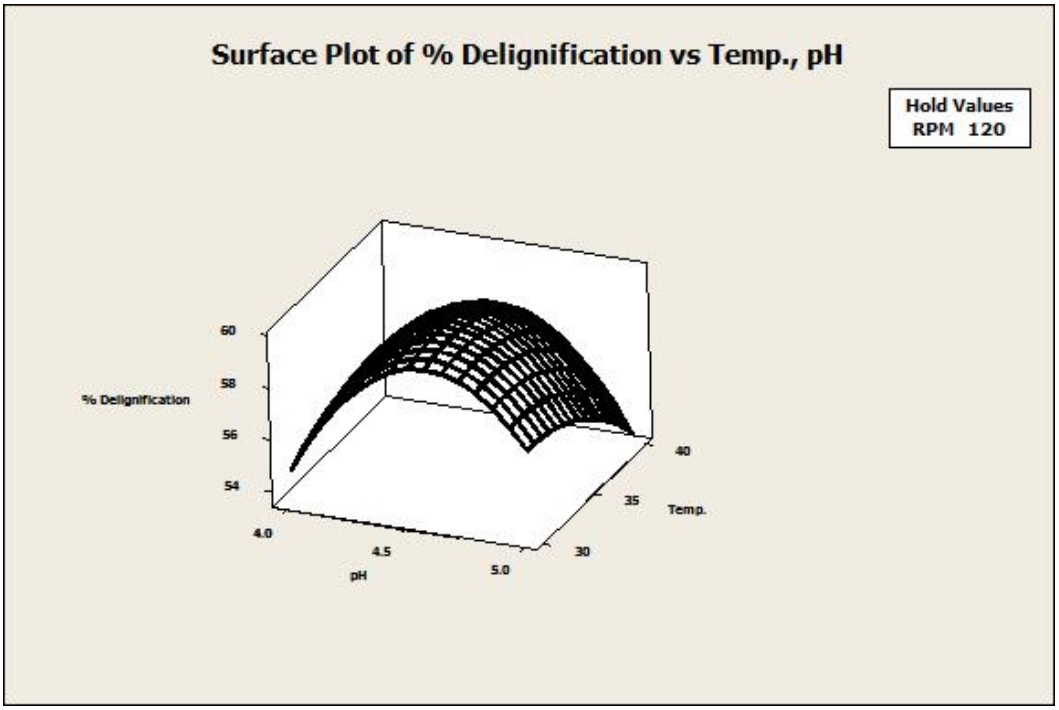


Figure 5.19: Response surface plots showing the effect of pH and temperature on the pretreatment of cotton gin waste

5.2.4 FTIR, XRD and SEM analysis of untreated and pretreated cotton gin waste

Similar to previous chapter, FTIR, XRD and SEM study was done to demonstrate the removal of lignin by analysing the change in fractional group (FTIR), crystallinity (XRD) and surface topography (SEM). The characteristics peaks of cellulose and lignin are

obtained at 1700 cm^{-1} to 1750 cm^{-1} and 1513 cm^{-1} are belong to the untreated sample and no such peaks were observed in pretreated sample (figure 5.20).The absence of peaks may be due to the reduction of compounds rich in carbonyl (C=O) groups (mostly lignin), removal of hemicellulose and other extractives during the pretreatment process. The absorption band at 2729 cm^{-1} is attributed to the stretching vibrations of hydroxyl (OH) groups present in the untreated (control) sample. Furthermore, a difference in the intensity of absorption at ~2500 cm^{-1} band size was due to the difference in absorbed water content between untreated and pretreated samples. This can be explained by a change in the degree of inter molecular H-bonding between OH group of cellulose and water. It can be expected that there would be an increase in surface area and rearrangement of cellulose microfibrils which may provide a better accessibility to OH group by the enzymes in the pretreated sample as the similar study also supports our results [153]. The OH groups at 3345 cm^{-1} may be aliphatic compounds, primary and secondary alcohols found in cellulose, hemicellulose, and carboxylic acids in extractives [154,155].The shoulder near the OH stretching vibrations, 2854 cm^{-1}, is attributed to CH stretching vibrations and corresponds to the aliphatic moieties in polysaccharides (cellulose and survived hemicellulose) of treated sample. The bands in the 1451-1333 cm^{-1} and 1450-1357 cm^{-1} region in the untreated sample may be due to CH in-plane deformation of CH_2 groups while the peaks at 1157-1058 cm^{-1} are due to the linkage present in the cellulose in both the samples. The pure cellulose peaks were obtained at frequencies- 1431, 1372, 1318, 1281, 1164, 1059 and 897 cm^{-1}.

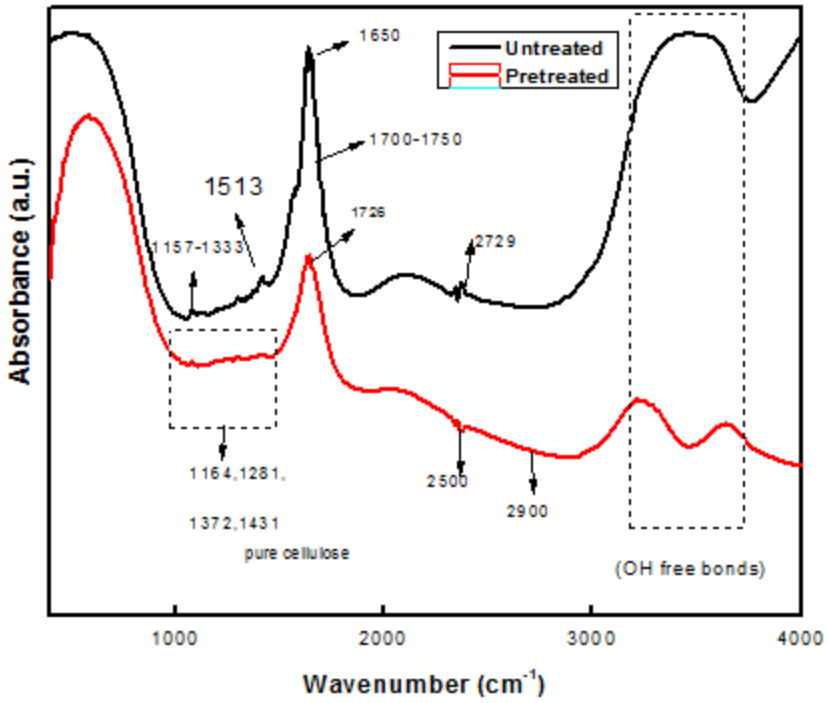


Figure 5.20: FTIR analysis of untreated and pretreated cotton gin waste

Although cellulose macrofibrils have been observed more profusely with increased pretreatment time, no change in the XRD was detected, as shown in figure 5.21. The XRD of untreated and pretreated samples exhibited similar crystalline patterns. The widths at half height of the peaks at 2θ =17º and 26º were similar for all samples except a higher haziness in untreated sample, which suggested similarity in crystallite sizes [141].The cellulose crystallinity value of an untreated sample of cotton gin waste was 18.36% while that of the pretreated sample was 23.94% suggesting an improvement in crystallinity of the sample. The hydrogen bonding holds the adjacent chain in place relative to one another. The crystallinity of the pretreated sample was increased due to the removal of lignin and hemicellulose (both of which extend amorphousness to the material) [156]. It is expected that amorphous region present in between the regular crystalline region are subjected to enzyme attack, and the removal of the amorphous region exposed improvement in crystallinity as observed in the pretreated biomass.

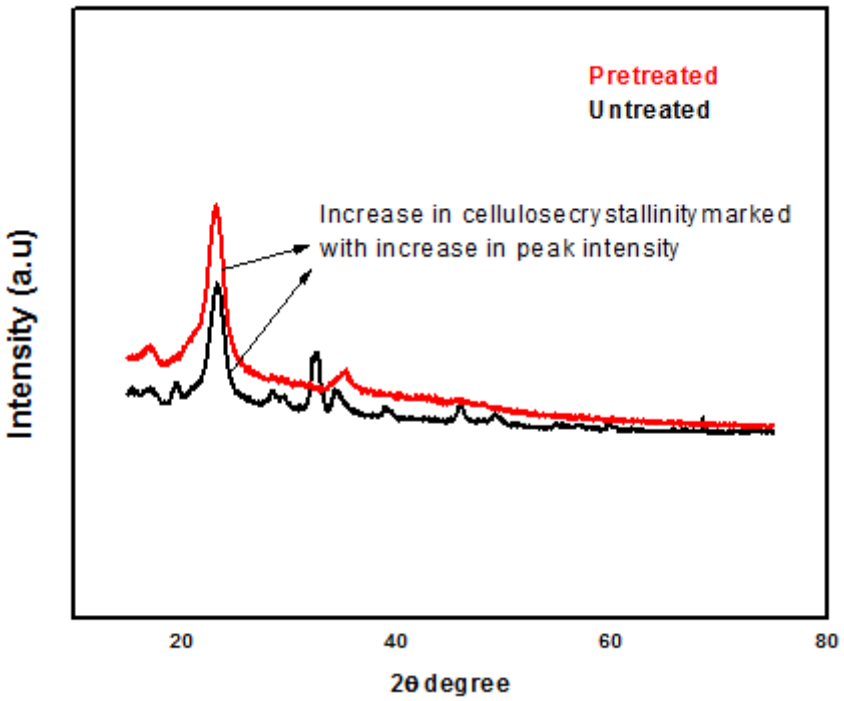


Figure 5.21: XRD analysis of untreated and pretreated cotton gin waste

Scanning electron micrographs (SEM) showed the morphology of cellulose and hemicellulose fiber with different severities as indicated in figure 5.22.The untreated sample shows compact fibers distributed over the whole region. Whereas, the pretreated biomass depicts partially degraded etched fibers indicating the influence of enzyme treatment. The figure shows that some macrofibrils remain separated, whereas other macrofibrils got agglomerated in pretreated biomass. This indicates an enhancement of surface area due to the removal of lignin and its associated compounds such as hemicellulose. A significant change in surface property towards favorable interaction with enzyme has occurred due to the cleavage of the amorphous region of cellulose with retention of the crystalline fraction by pretreatment. Additionally, lignin removal from pretreated sample increases the degree of crystallinity in compare to untreated sample [157].

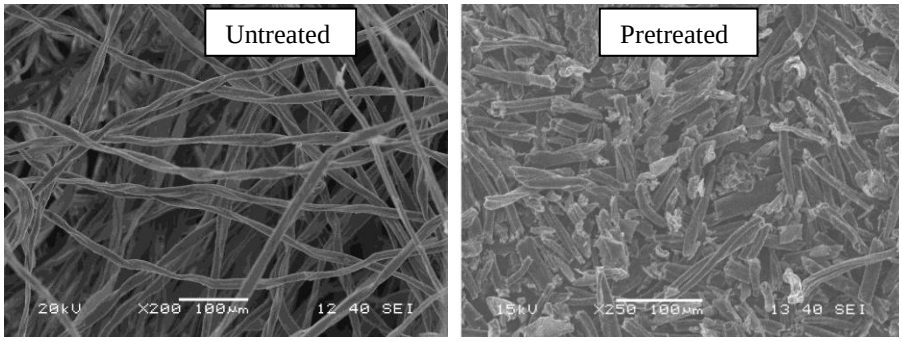


Figure 5.22: SEM images of untreated and pretreated cotton gin waste

5.2.5 Enzymatic hydrolysis of fungal pretreated cotton gin waste

The synergistic effect on multiple forms of cellulose and hemicellulose-degrading enzymes has been reported for achieving higher sugar release in hydrolysis [134,158]. Therefore, the conversion of cellulose and hemicellulose present in fungal pretreated biomass to fermentable sugar was done by hydrolysis using a mixture of cellulase, xylanase and β-glucosidase enzyme as depicted in figure 5.23. The hydrolysis experiment was carried at 50ºC, pH 5.5 and 150rpm [70]. During the progress of enzymatic hydrolysis, a steady increase in sugar concentration was observed till 64h, which on prolonged incubation remained almost constant. The maximum saccharification yield of 563.25 g/g and the total C5 and C6 sugar of 30.15 g/l with 56% (w/w) saccharification were achieved after 64h of hydrolysis using *P.cinnabarinus* pretreated CGW. The corresponding values of individual sugar concentration were 9.53±0.33 g/l xylose, 20.18±0.56 g/l glucose and 0.45±0.54 g/l arabinose till 64h.

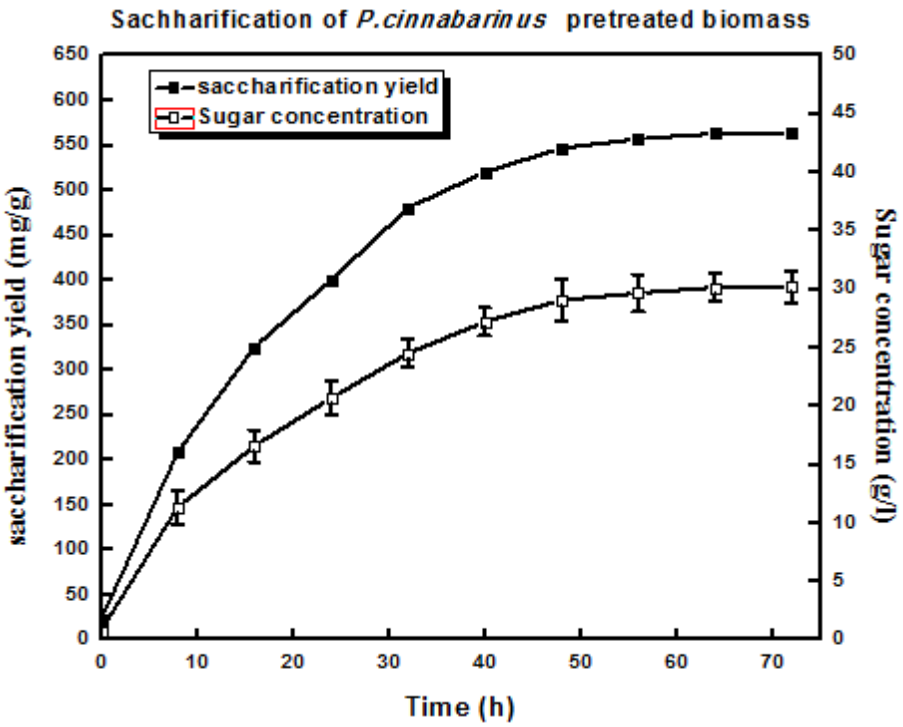


Figure 5.23: Enzymatic hydrolysis of *Pycnoporus cinnabarinus* pretreated cotton gin waste at 50^{o}C, pH 5 and 150rpm

5.2.6 Fermentation of enzymatic hydrolysate

The glucose and xylose-rich enzymatic hydrolysates (30.15 g/l) produced by the hydrolysis of pretreated biomass were fermented with the sequential use of *S. cerevisiae* and *P. stipitis* to produce bioethanol as the end product. The bioethanol production was increased with increase in fermentation time till 56h of fermentation and then decreased with further increase in time. The maximum 12.88 g/l ethanol concentration, 0.42 g/g ethanol yield, 83% theoretical yield and 1.06 g/l/h ethanol productivity were obtained after 56h of fermentation. The reduction in ethanol production beyond 56h of incubation may be due to the consumption of accumulated ethanol by the yeast [38] as shown in Table 14. When the ethanol starts accumulation in the medium, the yeast population might have consumed sugar and ethanol simultaneously as reported earlier [16]. The biomass concentration was also steadily increased with time and biomass yield of 0.31 g/g was obtained at 56h of fermentation (Table 14). The increased trend in yeast biomass during the progress of fermentation may be because of the utilization of the yeast extract present in fermentation medium till 64h and the yield which was then declined gradually due to the reaching of stationary phase of yeast strain [132].

Table 14**:** Fermentation of enzymatic hydrolysate by sequential use of *S. cerevisiae* and *P. stipitis* yeast strains at 200rpm, 30^{o}C and pH 5.5

Time (h)	Ethanol conc.(g/l)	Sugar (g/l) Consumption	Ethanol yield (g/g)	Biomass (g/l)	Biomass yield (g/g)
0	0.12	30.15	0.02	0.25	0.1
8	8.53	11.52	0.28	3.37	0.14
16	9.67	10.31	0.30	4.22	0.17
24	11.32	6.46	0.37	5.05	0.19
32	11.81	5.83	0.39	5.74	0.21
40	12.34	5.05	0.40	6.06	0.24
48	12.55	4.57	0.41	6.80	0.29
56	12.88	4.03	0.42	7.81	0.31
64	12.80	3.71	0.42	7.83	0.31
72	12.76	3.55	0.41	7.65	0.30

5.3 Bioethanol production from cotton gin waste: Effect of mixed fungal pretreatment

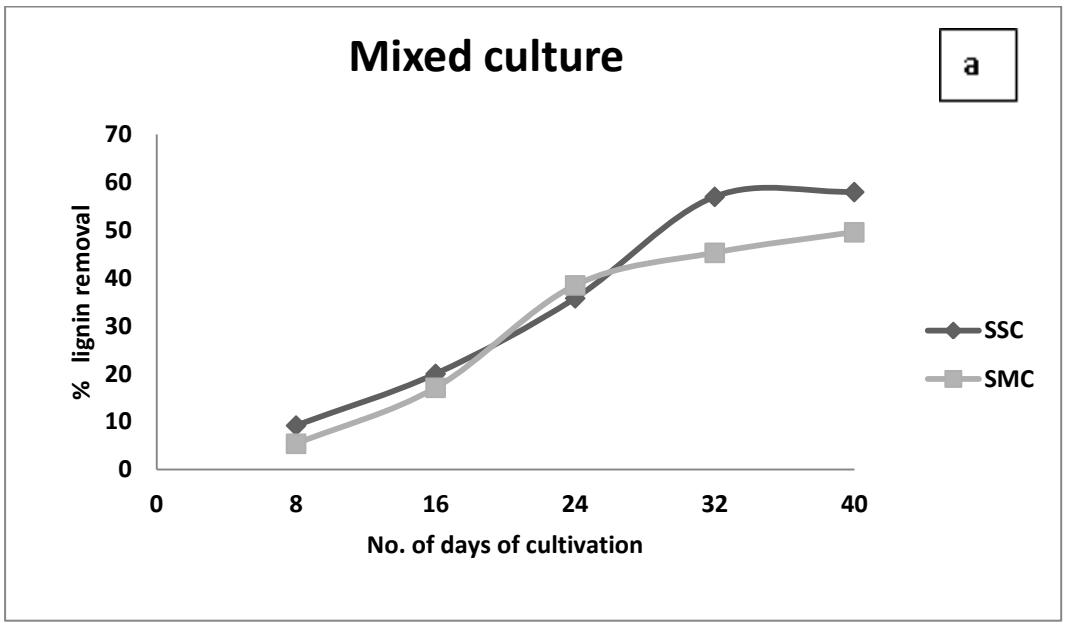
In has been reported that co-culture of some white rot fungi are more efficient for lignin-degradation than the individual culture [86,159]. It is further reported that the efficiency of lignin removal by mixed culture depends on various factors such as the type of species, mode of interaction between them and microenvironment [87]. However, no work has been reported so far on the use of mixed culture for the pretreatment of CGW. In the previous chapter, *Pycnoporus cinnabarinus* and *Trametes pubscens* were proven to be the most effective fungal strains producing high lignin degradation for the pretreatment of cotton gin waste.

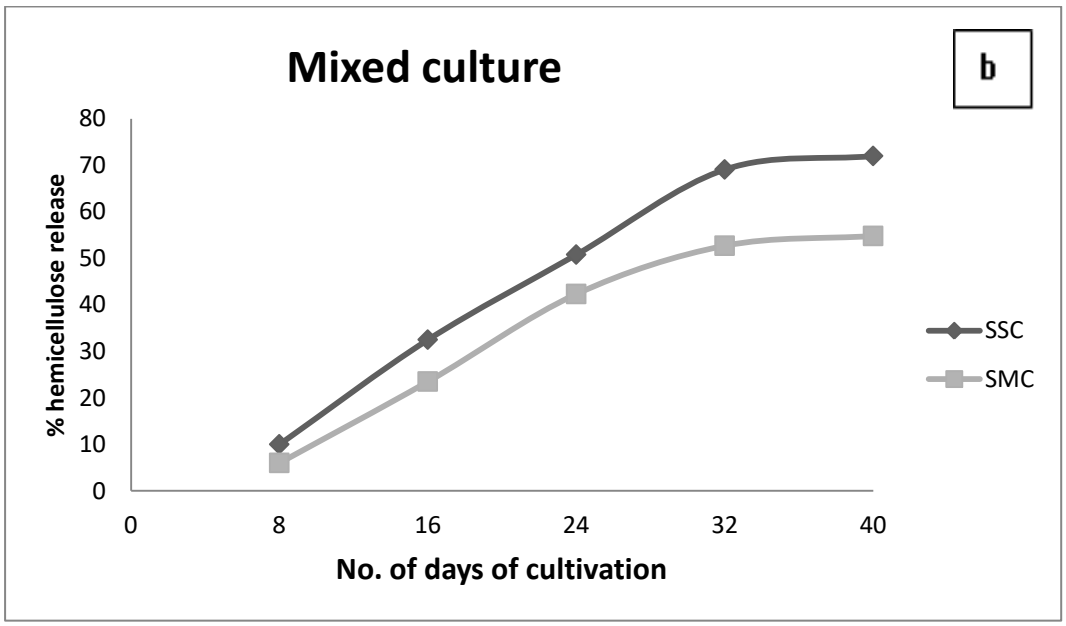
The present study investigates the efficiency of *Pycnoporus cinnabarinus* and *Trametes pubscens* on the delignification of cotton gin waste in a mixed culture media using solid and submerge state of cultivation with the aim of improving delignification of the cotton gin waste thereby increased the release of C5 and C6 sugar components. An effort was also given to optimizing the key pretreatment parameters by response surface model (RSM) based on central composite design (CCD). The untreated and pretreated biomass in terms of lignin degradation pattern was assessed by FTIR, XRD and SEM analysis for the structural and morphological changes of cotton gin waste. Furthermore, the efficiency of the pretreated CGW for enzymatic hydrolysis was examined and finally, the bioethanol production from hydrolysates was evaluated by the sequential use of yeast strains at optimum fermentation conditions. The result and discussion of the above experimental work are presented in this chapter.

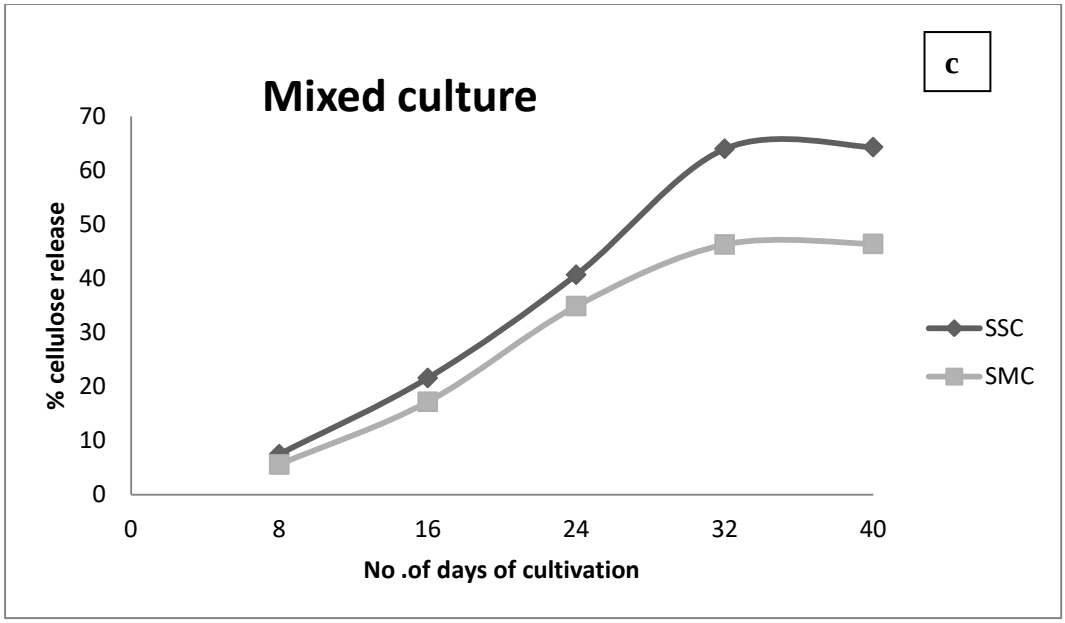
5.3.1 Pretreatment of cotton gin waste using mixed fungal culture

The ability of mixed culture of *Pycnoporus cinnabarinus* and *Trametes pubscens* towards the pretreatment of cotton gin waste in terms of lignin removal thereby releasing cellulose and hemicellulose during 40 days of cultivation is depicted in figure 5.24. An increased trend in degradation was observed till 32 days of cultivation and thereafter the rate of degradation started the decline. After 32 days lignin removal was observed as 57.5% and 48.6% using SSC and SMC respectively as shown in figure 5.24(a). The corresponding cellulose and hemicellulose release were achieved as 62.4% and 70 % in SSC, whereas their values in SMC were measured to be 46.2% and 52% respectively (figure 5.24 b & c).

Thus, the solid state cultivation degraded more lignin in comparison to SMC using the mixed culture of fungi. Furthermore, an improvement in delignification thereby increased cellulose and hemicellulose release using mixed culture than individual culture even the most efficient *Pycnoporus cinnabarinus* strain was obtained. This enhanced potentiality might be due to the potential synergistic actions of *Pycnoporus cinnabarinus* and *Trametes pubscens* on the pretreatment of cotton gin waste [159].







Figure

Figure 5.24: Effect of mixed fungal pretreatment on the release of cellulose (a), hemicellulose (b) and delignification (c) for 40 days of cultivation at 35°C, 100rpm and 4.5pH

5.3.2 Effects of wash and heat-wash pre-hydrolysis treatments

The delignified cotton gin waste was subjected to heat-wash treatment for better accessibility of biomass towards enzymatic hydrolysis. The heat-wash treatments showed the lignin removal of 0.5% and 0.4% in solid and submerge cultivation respectively. The corresponding cellulose and hemicellulose release was achieved as 0.2% and 0.5% in SSC, whereas their values in SMC were 0.3% and 0.8%. Previously heat-wash pre-hydrolysis treatment was reported to be beneficial for removing lignin derivatives and attached fungal mycelia on biomass, thereby increasing enzyme accessibility of cellulose and hemicellulose for hydrolysis [152].

5.3.3 Optimization of pretreatment parameters

Similar to previous chapter the pretreatment of cotton gin waste using fungal mixed culture was optimized by optimizing the key pretreatment parameters such as pH, temperature and shaking speed and the result is shown in Table 15. The delignification was achieved in the range of 50.1- 63.2% with the different response of pretreatment parameters. 63.2% was found to be the highest delignification achieved at optimum pretreatment condition of 4.5, 122 rpm and 35°C.

Table 15: Experimental design for the pretreatment of cotton gin waste using fungal mixed culture in terms of coded factor and its effect on delignification

Std Order	pH	Temp°C	RPM	% Delignification
1	4	30	100	59.5
2	5	30	100	58.0
3	4.	40	100	57.7
4	5	40	100	56.0
5	4	30	140	55.0
6	5	30	140	54.6
7	4	40	140	51.4
8	5	40	140	52.0
9	4	35	120	50.9
10	5	35	120	56.8
11	4	30	120	59.5
12	4	40	120	50.1
13	4	35	100	57.5
14	4	35	140	56.9
15	4.5	35	120	63
16	4.5	35	120	63.01
17	4.5	35	120	63.2
18	4.5	35	120.	63
19	4.5	35	120	63.2
20	4.5	35	120	59.6

In this study, the regression model equation showing the effect of delignification by all the three variables and their interaction was represented below in terms of coded factor:

$$Y = 62.62 + 0.37A_1 - 0.29A_2 + 0.66A_3 - 3.42A_1^2 - 2.52A_2^2 - 0.67A_3^2 + 0.36A_1A_2 - 0.78A_1A_3 - 0.13A_2A_3$$

Where, A_1, A_2 and A_3 represent pH, temperature and rpm respectively, and Y represents the experimental response. The ANOVA analysis of the quadratic regression for mixed fungal pretreatment of cotton gin waste has been summarized in Table 16.

Table 16: ANOVA analysis of RSM model for fungal pretreatment of pretreated CGW

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	181.194	181.194	20.132	50.87	0.000
Linear	3	6.566	6.566	2.1887	5.53	0.017
Square	3	168.465	168.465	56.1548	141.89	0.000
Interaction	3	6.164	6.164	2.0546	5.19	0.020
Residual Error	10	3.958	0.3958	0.3958		
Lack-of-Fit	5	3.824	3.824	0.7649	28.68	0.001
Pure Error	5	0.133	0.133	0.0267		
Total	19	185.152				
R^2 = 0.9786						

DF= degree of freedom, SS= sum of squares, MS= mean sum of squares, F= Fisher's F value and P= probability.

The square and interaction effects between the variables were found to be statistically significant with a low P- value (0 and 0.02). Although the square terms of the model shows more significance with the highest F-value of 141.89 by square effects. The regression model for the pretreatment of cotton gin waste has shown an effective F-value (50.87) and a very low probability value (< 0.001) representing the significance of the model [66]. The R^2 value obtained as 0.9786, which is close to 1 and hence justifies the robustness of the model that sufficiently fits to data. Figure 5.25 shows the interaction of temperature and rpm at a constant pH, where as figure 5.26 shows the interaction between pH and rpm at constant temperature on lignin degradation of the pretreated sample. The three-dimensional plots show that the temperature of 35°C and shaking speed 120 rpm caused an increase in lignin degradation (%), yielding a maximum delignification of 63.01% after 35 days of solid-state cultivation. However at a constant temperature, the interaction between shaking speed and pH yielded the maximum value of delignification as 63.2%. In figure 5.27, the optimization has been performed with variation in

temperature and pH, with constant rpm which shows less response in comparison to other [160,161]. From the experimental data, the above statistical model represents the optimum predicted condition of pH, shaking speed and temperature as 4.5, 122 rpm and 35°C respectively with a high percentage of delignification. In order to check the reliability of the predicted response, the triplicate experiment was performed under optimum predicted conditions. From this design of experiments, the maximum release of cellulose and hemicelluloses were found to be 63.7% and 71.02%. Whereas, delignification efficiency was 63.2%, which is a good agreement with the predicted value of the model. Therefore, we conclude that solid state of cultivation shows effective delignification at optimized process condition established by response surface model.

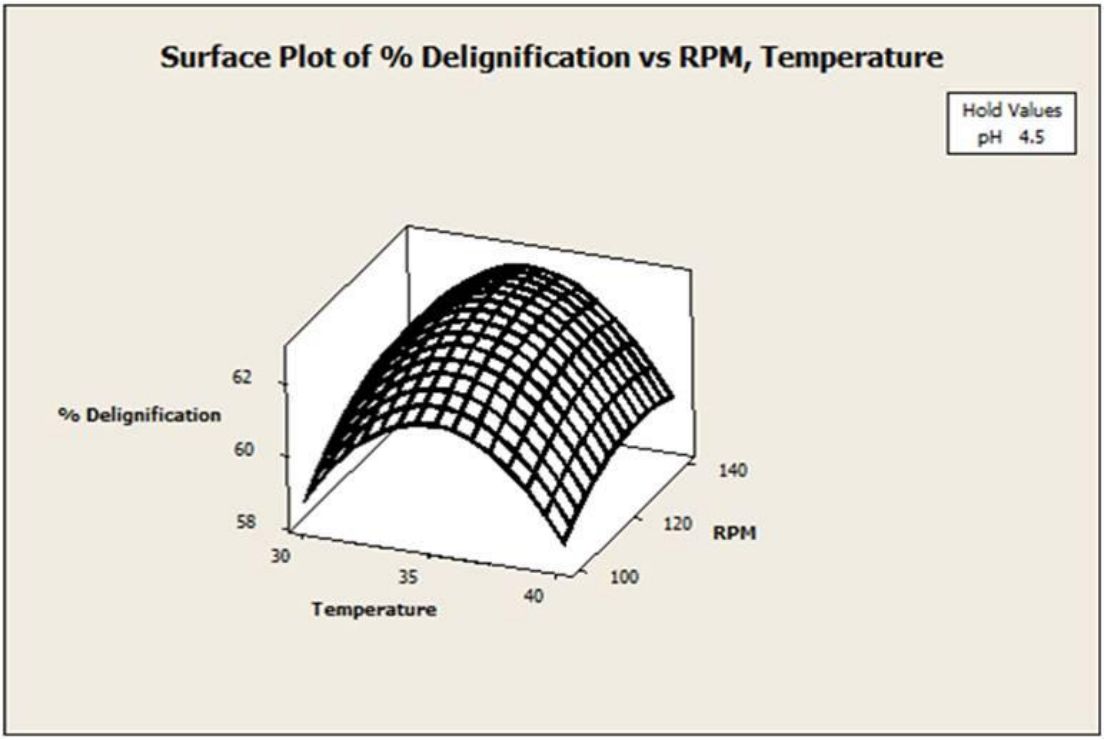


Figure 5.25: Response surface plots showing the effect of temperature and shaking speed on the pretreatment of cotton gin waste

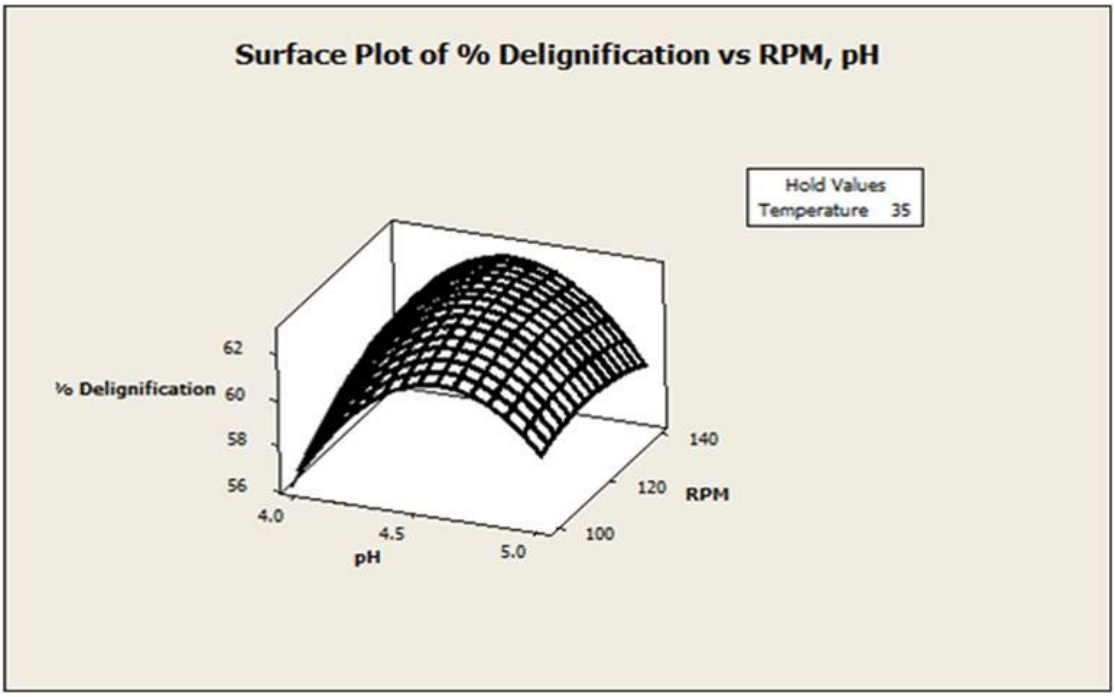


Figure 5.26: Response surface plots showing the effect of pH and shaking speed on the pretreatment of cotton gin waste

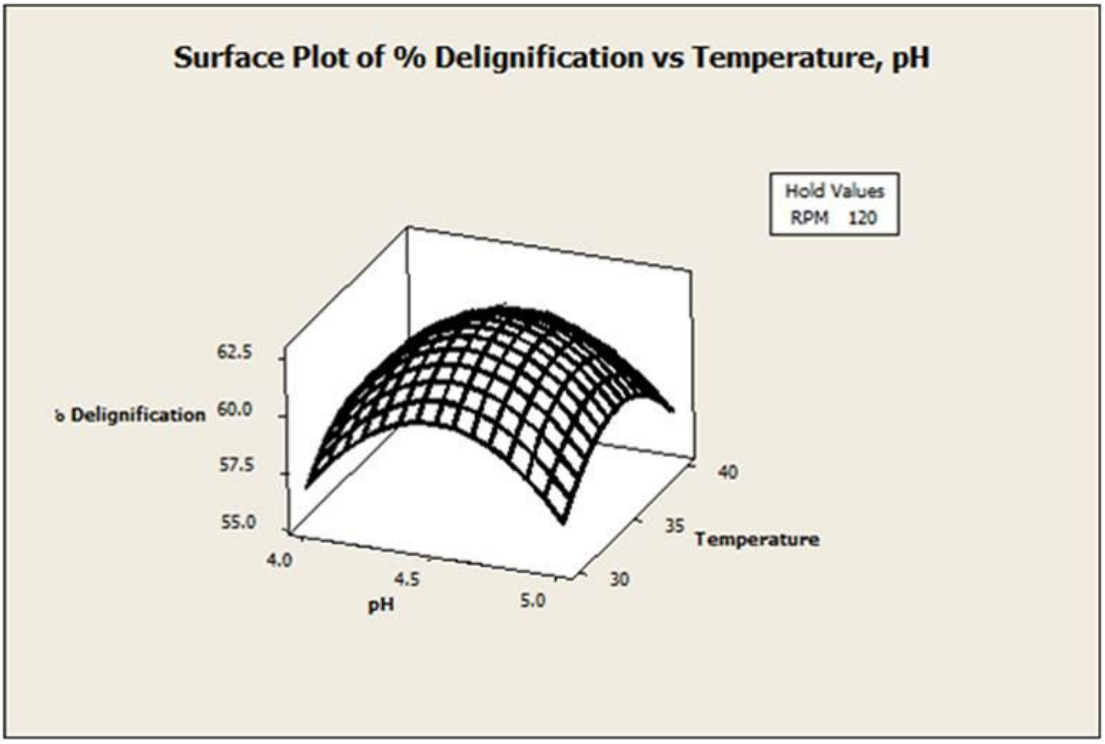


Figure 5.27: Response surface plots showing the effect of pH and temperature on the pretreatment of cotton gin waste

5.3.4 FTIR, XRD and SEM analysis of untreated and pretreated cotton gin waste

The characteristic peaks of lignin at 1700 cm^{-1} to 1750 cm^{-1}, 1726 and 1512 cm^{-1} belongs to the untreated biomass and no such peaks were noticed with the pretreated sample as depicted in figure 5.28 [132,141]. The absorption band at 3393 cm^{-1} is attributed to the stretching vibrations of hydroxyl (OH) groups present in the untreated sample [132]. Furthermore, a difference in intensity of absorption at ~2500 cm^{-1} band size is due to the difference in absorbed water content between untreated and pretreated samples representing the degree of inter molecular H-bonding between OH group of cellulose and water [141]. It can be expected that there would be an increase in surface area and rearrangement of cellulose microfibrils which may provide a better accessibility to OH group by the enzymes on the pretreated sample [153]. The OH groups at 3345 cm^{-1} may include aliphatic compounds, primary and secondary alcohols found in cellulose, hemicellulose and carboxylic acids in extractives [132]. The shoulder near OH stretching vibrations, 2900 cm^{-1}, is attributed to CH stretching vibrations and corresponds to the aliphatic moieties in polysaccharides (cellulose and survived hemicelluloses) of the treated sample. The pure cellulosic peaks are obtained at frequencies- 1431, 1372, 1318, 1281, 1165, 1059 and 897 cm^{-1} [141].

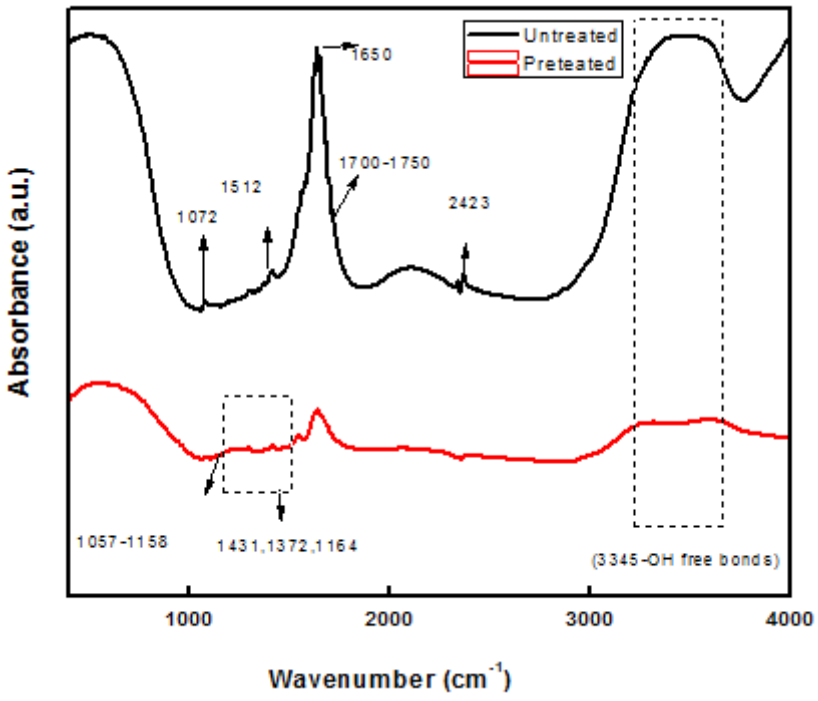


Figure 5.28: FTIR spectra of untreated and pretreated cotton gin waste

Cellulose macrofibrils have been observed more prominently with increased in pretreatment time as shown in figure 5.29. XRD for untreated and pretreated samples exhibited similar crystalline patterns. The widths at half height of the peaks at 2θ =17º and 26º were similar for all samples except a higher haziness in untreated sample, which suggests similarity in crystallite sizes [141].The cellulose crystallinity of the pretreated cotton gin waste was 25.1% measured as showing a significant improvement in crystallinity of the sample. This may be due to the removal of lignin and hemicellulose [162]. It is expected that amorphous region present in between the regular crystalline region is subjected to enzyme attack after pretreatment.

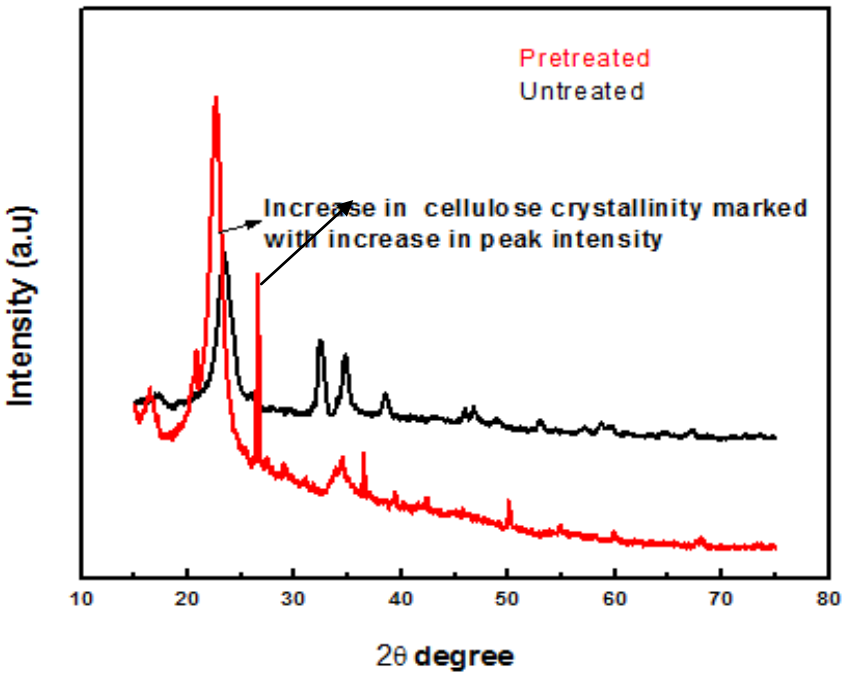


Figure 5.29: XRD of untreated and pretreated cotton gin waste

SEM micrograph (figure 5.30) reveals the morphology of cellulose and hemicellulose fiber with different severities. The untreated sample shows the compact fibers distributed over the whole region. Whereas the pretreated sample possesses partially degraded etched fibers indicating the influence of fungal treatment that resulted an enhanced surface area and significant change in the surface property towards favorable interaction with an enzyme that has occurred due to pretreatment was resulting in cleavage of the amorphous region of cellulose with retention of crystalline fraction. Additionally, lignin and hemicelluloses removal from the pretreated sample have increased the degree of crystallinity [157].

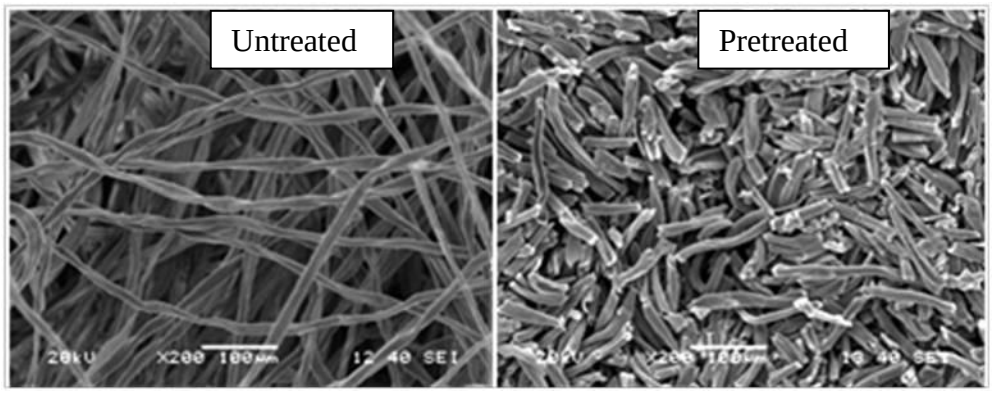


Figure 5.30: SEM analysis of untreated and pretreated cotton gin waste

5.3.5 Enzymatic hydrolysis of delignified cotton gin waste

The conversion of cellulose and hemicelluloses fractions present in pretreated biomass to C5 and C6 sugars during 72h of enzymatic hydrolysis is shown in figure 5.31. The concentration and yield of sugars were observed to be increased with hydrolysis time up to 64h and then a decline in sugar production was observed. 59% (w/w) saccharification was achieved to by releasing 32.25 g/l total sugar and the maximum saccharification yield was 590.25 g/g after 64h. The corresponding concentration of individual sugar component was measured to be 9.62±0.33 g/l xylose, 22.04±0.34 g/l glucose and 0.65±0.11 g/l arabinose. The decline in hydrolysis rate beyond 64h of hydrolysis may be due to the increasing resistance of the substrate during the course of hydrolysis may be due to other factors [69]. In hydrolysis, a higher sugar concentration was obtained using mixed fungal pretreated biomass in comparison to individual fungal pretreatment.

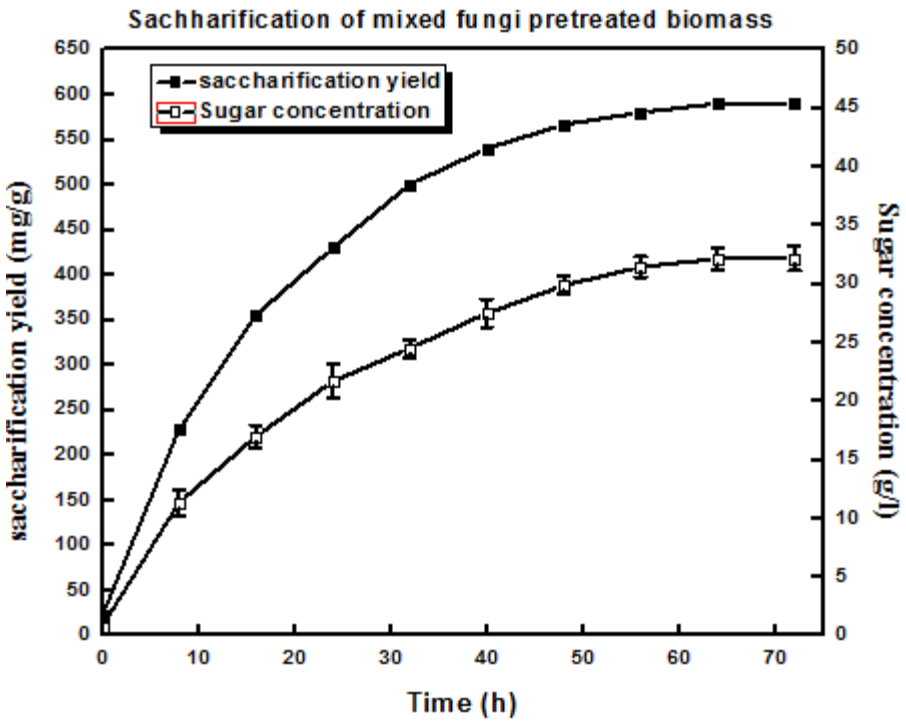


Figure 5.31: Enzymatic saccharification of mixed fungal pretreated cotton gin waste at waste at 50^{o}C, pH 5 and 150rpm

5.3.6 Fermentation of enzymatic hydrolysis

The fermentation of 32.25 g/l sugar hydrolysate was performed by a sequential use of *S. cerevisiae* and *P.stipitis* yeast strains and the experimental result is depicted in Table 17. The bioethanol production was increased with fermentation time up to 56h, which became almost constant with a prolonged period. A decline in ethanol concentration was obtained after 56h of fermentation, may be due to the yeast strains consumed accumulated ethanol [145]. The maximum ethanol concentration of 14.08 g/l, 0.43 g/g ethanol yield with 1.25 g/l/h productivity were obtained. The maximum theoretical yield of ethanol was calculated as 85%. The maximum biomass yield was found to be 0.31 g/g on 48h (Table.14). The increased trend in yeast biomass during the progress of fermentation may be because of the utilization of yeast extract present in fermentation medium till 48h which was then declined gradually till the stationary phase of yeast strain is reached [70]. Thus, it has been demonstrated that the pretreatment using mixed culture is more efficient achieving higher ethanol production than the pretreatment using individual strain like *Pycnoporus cinnabarinus.*

Table 17: Fermentation of enzymatic hydrolysate by sequential use of *S. cerevisiae* and *P. stipitis* of yeast strains at 200 rpm, 30^{o}C and pH 5.5

Time (h)	Ethanol conc.(g/l)	Sugar (g/l) Consumption	Ethanol yield (g/g)	Biomass (g/l)	Biomass yield (g/g)
0	0.29	32.25	0.02	0.26	0.05
8	9.82	12.08	0.30	3.09	0.18
16	11.35	9.24	0.35	3.52	0.29
24	12.47	7.01	0.38	4.75	0.23
32	12.95	6.34	0.40	5.42	0.26
40	13.24	5.93	0.41	6.80	0.29
48	13.64	5.05	0.42	7.94	0.31
56	14.04	4.03	0.44	7.96	0.31
64	14.07	3.84	0.44	7.87	0.30
72	13.90	3.53	0.43	7.60	0.29

5.4 Bioethanol production from cotton gin waste: Effect of fungal strain isolated from the soil of cotton mill

We hypothesized that the isolation of lignocellulosic microorganisms that are adapted to the lignocellulosic-rich environment while exhibiting strong lignin degradation ability may be beneficial for biotransformation of cotton gin waste to value added product like bioethanol [86]. Therefore, the research work in this chapter focuses on the isolation of fungal strains from the soil of a dumping area of cotton ginning mill. The efficiency of the isolated strains for the pretreatment of cotton gin waste was investigated. The potential fungi were selected on the basis of their lignolytic activity and growth capacity on cotton gin waste. Further assessment was done to find the ability of the isolated strains for delignification thereby release of sugar components, which were further accessed for hydrolysis and subsequently fermentation to produce bioethanol from cotton gin waste. This chapter describes the results and discussion on the above research work carried out.

5.4.1 Isolation and screening of microorganisms

The fungal strains were isolated from the soil collected from the dumping area of the cotton mill which is shown in figure 5.32.

Figure 5.32: Isolation of microorganisms from soil of the dumping area of Shree Ambica Agro Industries Ltd. cotton mill

Isolated fungal strains were separated by serial dilution and cultured on potato dextrose agar (PDA) plates for 5-7 days of incubation. The growth pattern of two fungi is depicted in figure 5.33 (a & b).

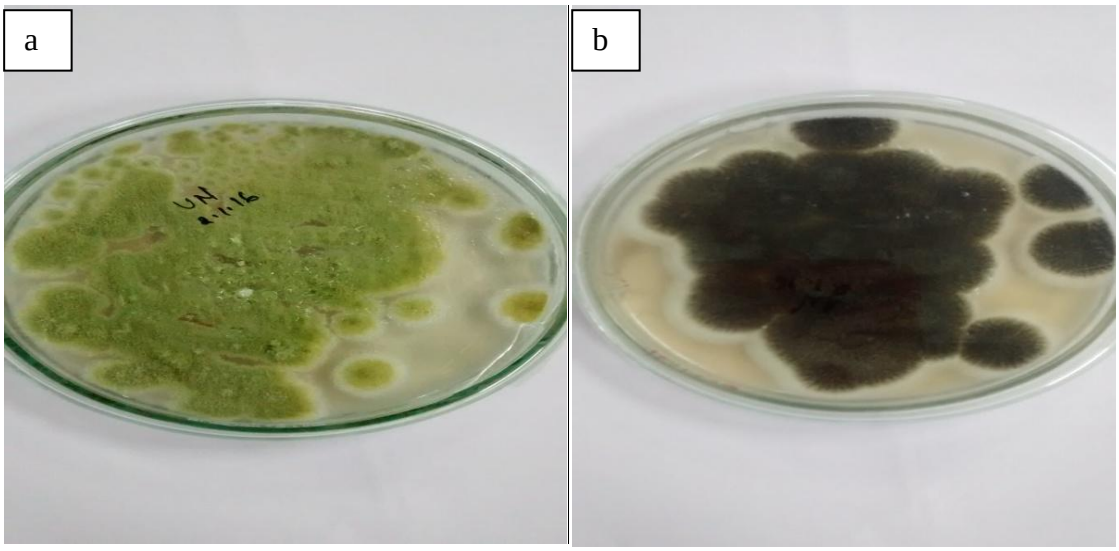


Figure 5.33: Showing the images of pure isolated fungal culture (a) UNF1 fungus and (b) UNF2 growth in PDA Agar media

Selection of effective lignolytic fungi was done on the basis of colony formation and zone of activity tests. The zone of activity results for identifying ligninase positive fungi are depicted in figure 5.34 (a & b). The zone of activity of UNF1 and UNF2 fungi were measured as 77 mm and 58 mm respectively. The higher zone of activity represents the higher lignolytic activity of fungal strains. As indicated in figure 5.34 (a & b), UFN1 showed greater ligninase positive activity in comparison to UNF2. Further, these two fungal species were selected and used for pretreatment study. The ability of the fungal strains to secrete extracellular laccase was visualized as intense reddish brown color in the medium around the fungal colonies and was taken as the positive reaction to the lignolytic activity [128,163,164]. The presence of brick red color around the mycelium was considered as guaiacol oxidizing laccase secreting organism.

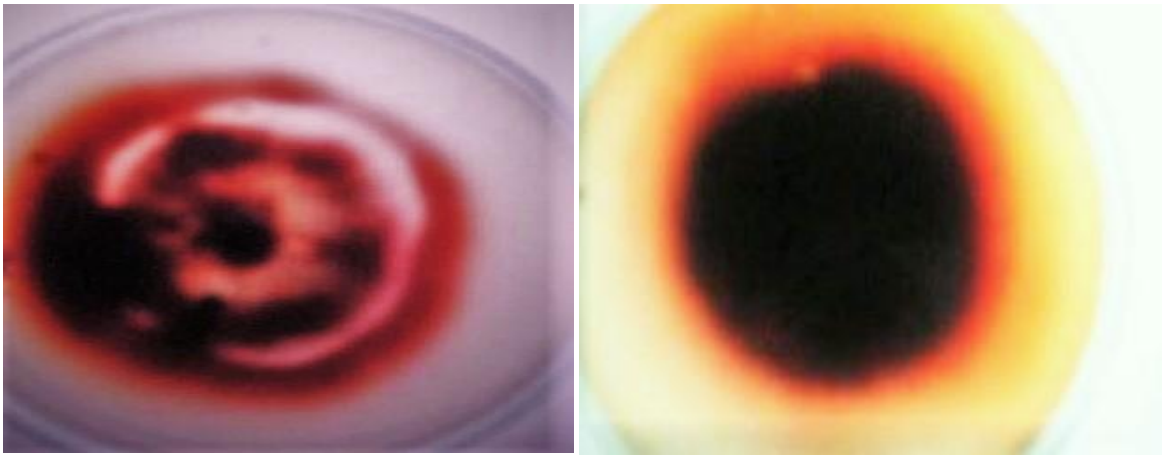
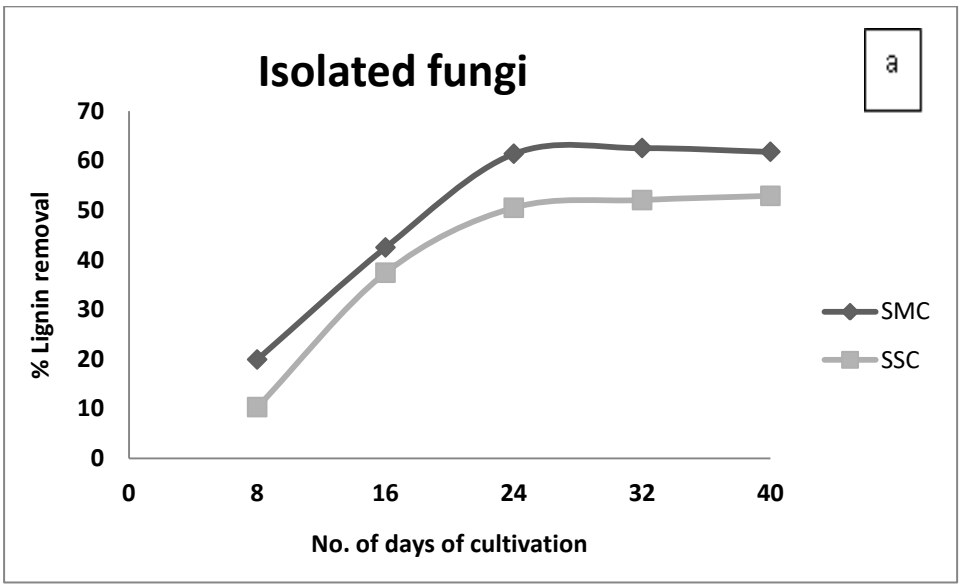
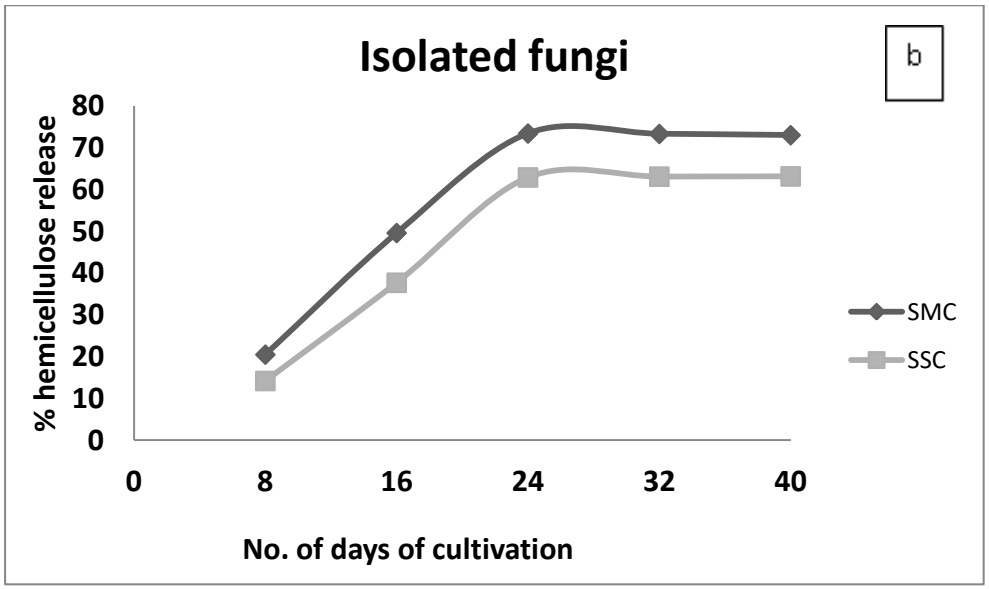


Figure 5.34: (a) UNF1 Zone of activity-70 mm (b) UNF2 zone of activity-58 mm

5.4.2 Pretreatment of cotton gin waste using isolated fungal strains

Initially, the pretreatment was carried out with both isolated UNF1 and UNF2 fungi and the respective 56.4% and 49% delignification was obtained in submerge state of cultivation. Further, the detailed study of pretreatment using UNF1 fungi showed 56.4% lignin removal in SSC and 61% in SMC. The cellulose and hemicellulose release after 24 days of the experiment (figure 5. 35 a, b & c) 65% and 72.81% in SMC, whereas their values in SSC were 55.2% and 62.7% respectively. Though the experiment was carried out for 40 days of cultivation, there was no significant improvement in lignin removal after 24 days of the cultivation which may be due to the growth rate of fungi. However, higher lignin removal efficiency was obtained with isolated fungus than the mixed culture of *Pycnoporus cinnabarinus* and *Trametes pubscens.*





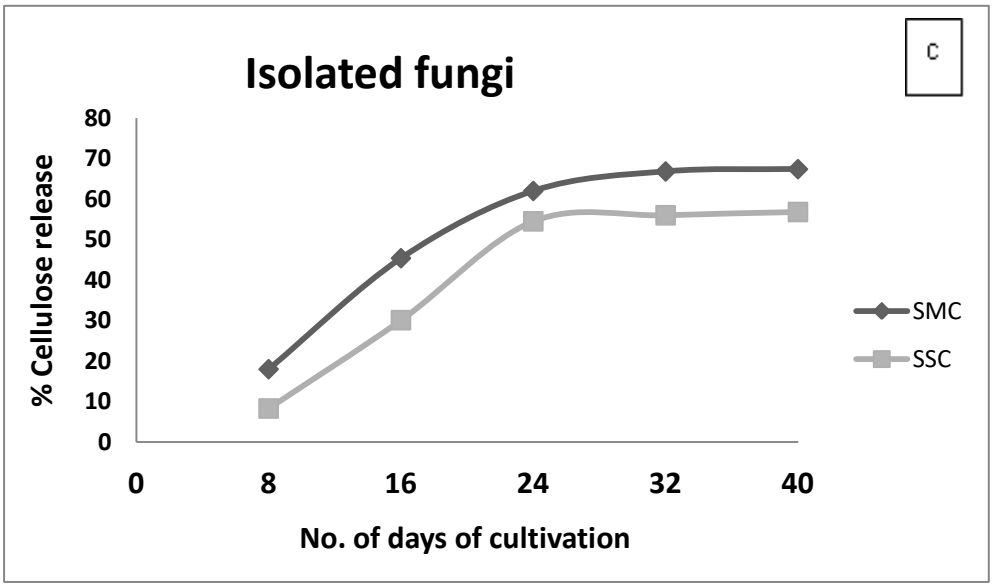


Figure 5.35: Effect of pretreatment on the release of cellulose (a), hemicellulose (b) and delignification (c) using isolated fungi UNF1 at 35^{o}C, 100 rpm and 4.5pH

5.4.3 Identification of isolated fungi

The phenotypic characterization of fungus UNF1 identified the strain belongs to *Aspergillus flavus*. Whereas UNF2 is *Aspergillus niger* as identified by phenotypic and morphological analysis in the laboratory. The microscopic analysis of the spore structure of the fungi is shown in figure 5.36 and 5.37.

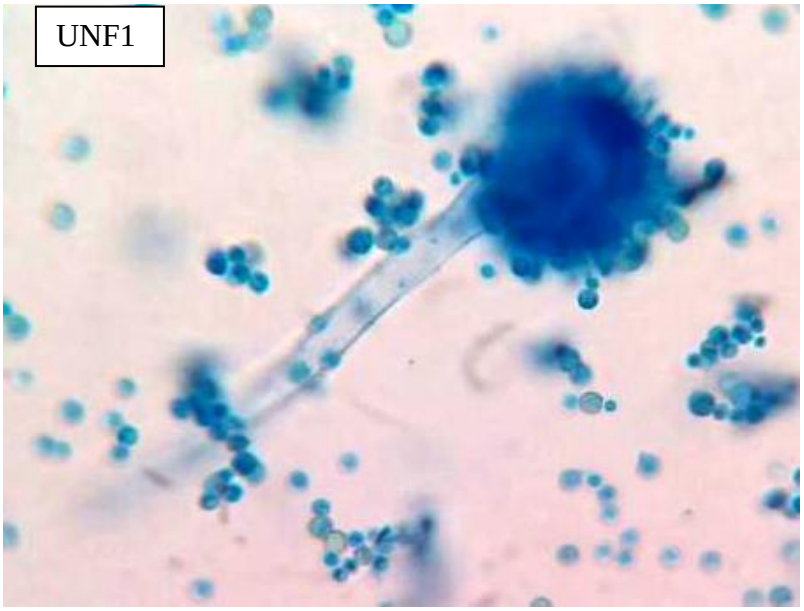


Figure 5.36: Microscopy structure analysis of the spore of fungi UNF1

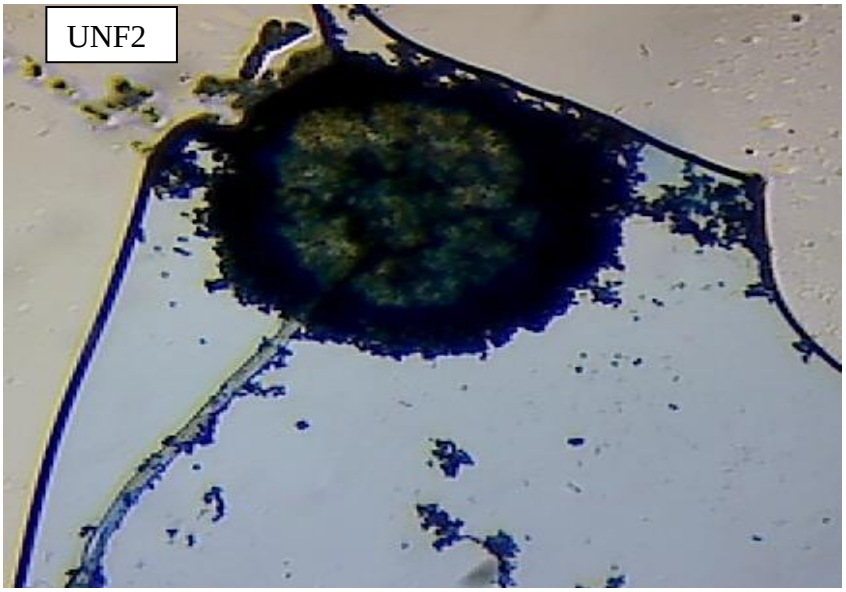


Figure 5.37: Microscopy structure analysis of the spore of fungi UNF2

5.4.4 Effects of wash and heat wash pre-hydrolysis treatments

The removal of lignin from fungal treated cotton gin waste by solid and submerge cultivation was slightly improved showing the respective 0.3% and 0.4% of delignification. The corresponding cellulose and hemicellulose release were obtained as 0.1% and 0.2 % in SMC, whereas their values in SSC were 0.3% and 0.2% respectively.

5.4.5 Optimization of pretreatment parameters

Similar to the previous chapter, RSM study based on central composite design (CCD) involving three individual parameters such as pH, temperature and shaking speed was performed for achieving improved delignification outcome as shown in table 18.

Table 18: Experimental design for the fungal pretreatment of cotton gin water in terms of coded factor and its effect on delignification

Std Order	pH	Temp°C	RPM	% Delignification
1	4	30	100	59.5
2	5	30	100	58.0
3	4.	40	100	57.7
4	5	40	100	56.0
5	4	30	140	58.0
6	5	30	140	54.6
7	4	40	140	51.4
8	5	40	140	52.0
9	4	35	120	60.9
10	5	35	120	63.8
11	4	30	120	59.5
12	4	40	120	55.1
13	4	35	100	57.3
14	4	35	140	58.9
15	4.5	35	120	67..2
16	4.5	35	120	67.04
17	4.5	35	120	67.01
18	4.5	35	120.	67
19	4.5	35	120	67
20	4.5	35	120	62.6

The percentage of delignification was obtained in the range of 54-67.04 % at optimized process conditions. The mathematical equation relating % of lignin degradation to the

different independent variables and their interactions is shown below in terms of coded factor:

$$Y= 67.02 + 0.37A_1 - 0.44A_2 + 0.66A_3 - 5.02A_1^2 - 2.52A_2^2 - 0.80A_3^2 + 0.36A_1A_2 - 2.18A_1A_3 - 0.78A_2A_3$$

Where, A_1, A_2 and A_3 represent pH, temperature and rpm respectively and Y represents the experimental response. The individual action of all the three parameters studied, where the quadratic and interaction effects between the dependent variables were found to be significant from the regression model. Analysis of Variance (ANOVA) of the quadratic regression for fungal pretreatment of cotton gin waste has been summarized in Table19. The linear and square effects between the variables were found to be statistically significant with a same low P- value (0.00), although the linear terms of the model show more significance with respect to high F-value (30.93). The regression model for the pretreatment of cotton gin waste has shown the highest F-value (57.87) and a very low probability value (< 0.001) thus signifying the model [66]. The quality of the model was evaluated by the coefficient R^2 and its statistical significance was determined by an F-test. In the pretreated sample, the R^2 values obtained was as 0.9653 and hence justify the robustness of the model [132].

Table 19: ANOVA analysis of RSM model for biological pretreatment of pretreated cotton gin waste

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	289.470	289.470	32.163	30.93	0.000
Linear	3	179.930	179.930	59.977	57.67	0.000
Square	3	70.336	70.336	23.445	22.54	0.000
Interaction	3	39.204	39.204	13.068	12.57	0.001
Residual Error	10	10.400	10.400	1.040		
Lack-of-Fit	5	10.091	10.091	2.018	32.73	0.001
Pure Error	5	0.308	0.308	0.062		
Total	19	299.869				
R^2 = 0.9653						

DF= degree of freedom, SS= sum of squares, MS= mean sum of squares, F= Fisher's F value and P= probability

Figure 5.38 shows the interaction of temperature and rpm at a constant pH, where as figure 5.39 shows the interaction between pH and rpm at constant temperature on lignin degradation of the pretreated sample. The three dimensional plots show that the temperature at 36°C, pH at 5 and shaking speed at 110 rpm caused an increase in the lignin degradation (%), yielding a maximum delignification of 67.04 % by releasing 66% cellulose and 73.5% hemicellulose after 24 days of submerge state cultivation. However, at a constant temperature, the interaction between shaking speed and pH gives the maximum value of delignification. In figure 5.40 the optimization has been performed with a variation of temperature and pH, where as rpm is kept constant [160,161].

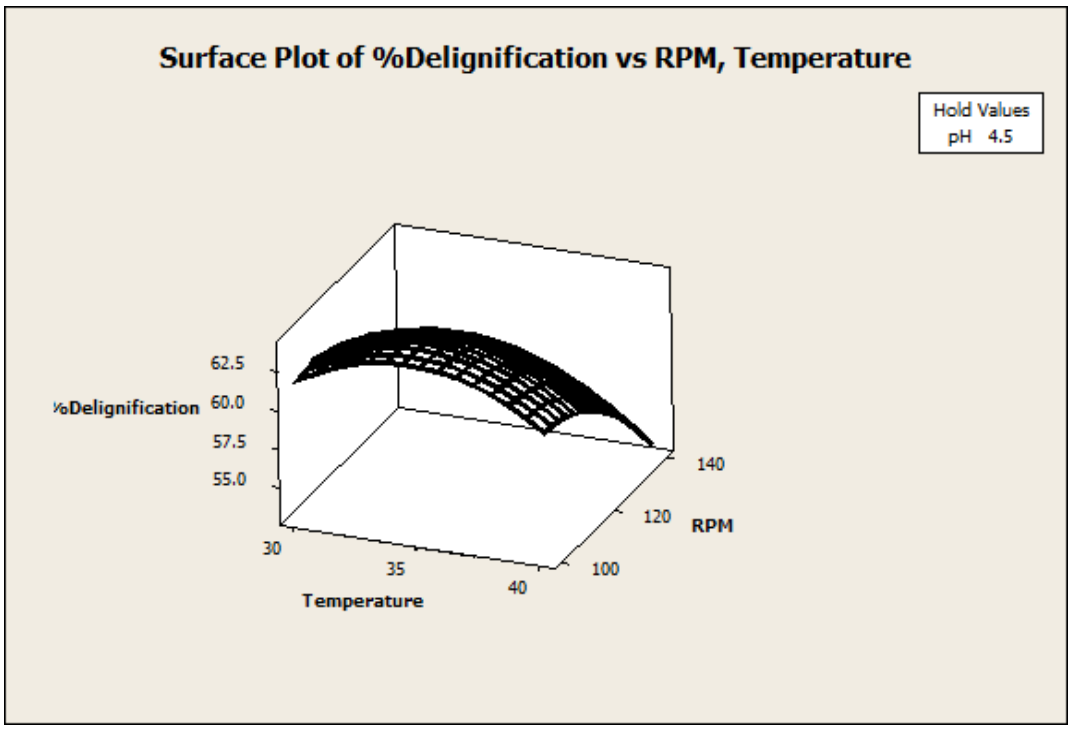


Figure 5.38: Response surface plots showing the effect of temperature and shaking speed on the pretreatment of cotton gin waste

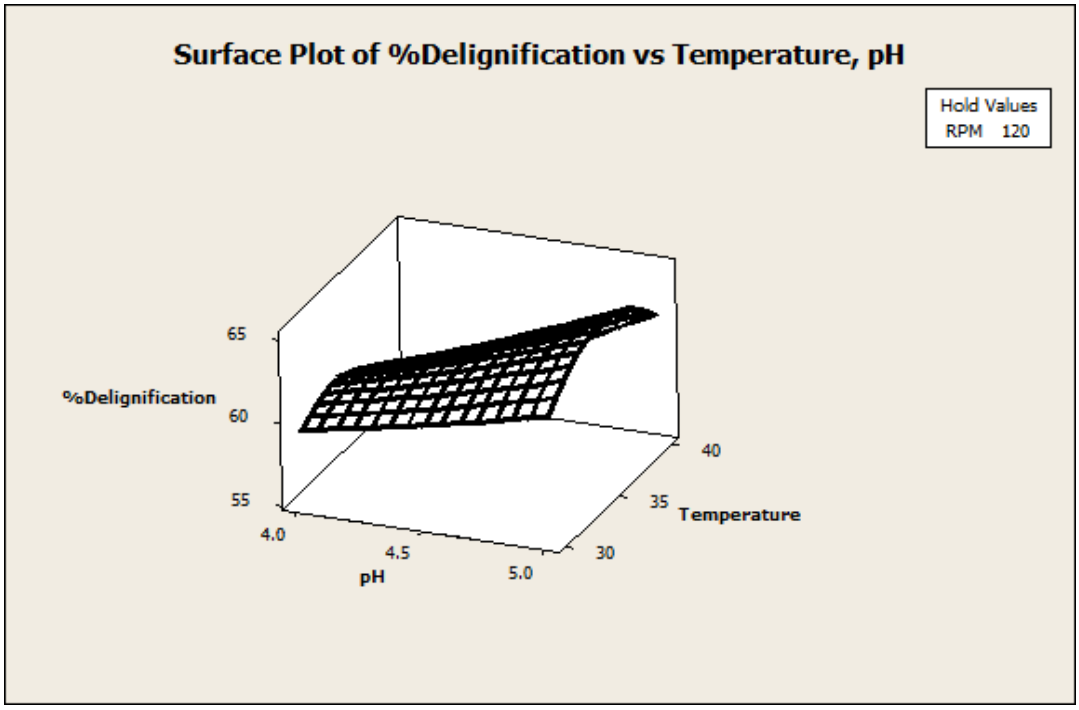


Figure 5.39: Response surface plots showing the effect of pH and shaking speed on the pretreatment of cotton gin waste

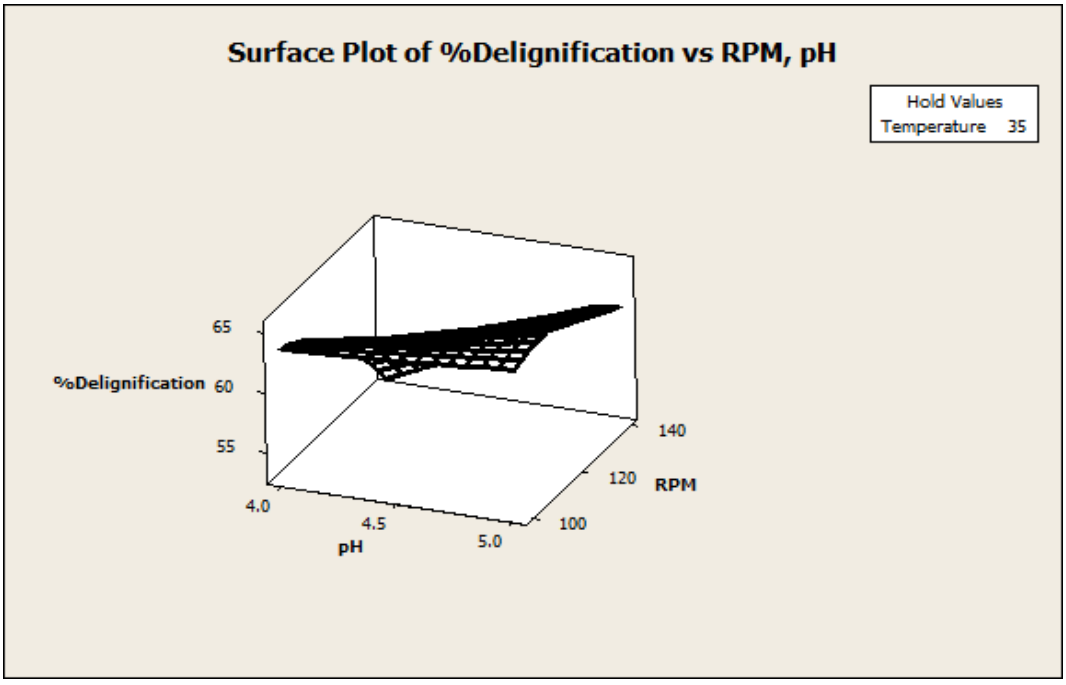


Figure 5.40: Response surface plots showing the effect of pH and temperature on the pretreatment of cotton gin waste

The statistical model suggests the optimum predicted the condition of pH, shaking speed and temperature as 4.5, 122 rpm and 35°C for a high percentage of delignification. In order to check the reliability of predicted response, the triplicate experiment was performed under optimum predicted conditions. From this study, the maximum release of cellulose and hemicelluloses was found to be 66% and 74.5%. Whereas delignification

efficiency was determined as 67.04%, this is a good agreement with predicted value. Therefore, we conclude that submerge state of cultivation providing delignification of the cotton gin, after optimization of the process parameters achieved through response surface model.

5.4.6 FTIR, XRD and SEM analysis of untreated and pretreated cotton gin waste

The FTIR image as shown in figure 5.41 the indicative peaks of cellulose and lignin obtained at 1700cm^{-1} to 1750 cm^{-1}, 1726 and 1512 cm^{-1} belong to the raw cotton gin waste, whereas no such peak was observed in the pretreated sample. The later may be due to the reduction of compounds rich in carbonyl (C=O) that is mostly lignin along with some amount C5 compound and other extractives during the pretreatment process. The absorption band at 3393 cm^{-1} is attributed to the stretching vibrations of hydroxyl (OH) groups in the untreated sample. Furthermore, a difference in intensity of absorption at ~2732 cm^{-1} band size was due to the difference in absorbed water content between untreated and pretreated samples. At 3354 cm^{-1} the OH groups were obtained in the pretreated sample. The bands at 1433, 1372, 1318, 1281, 1165, 1059 and 897 cm^{-1} frequency are corresponding to pure cellulosic peaks.

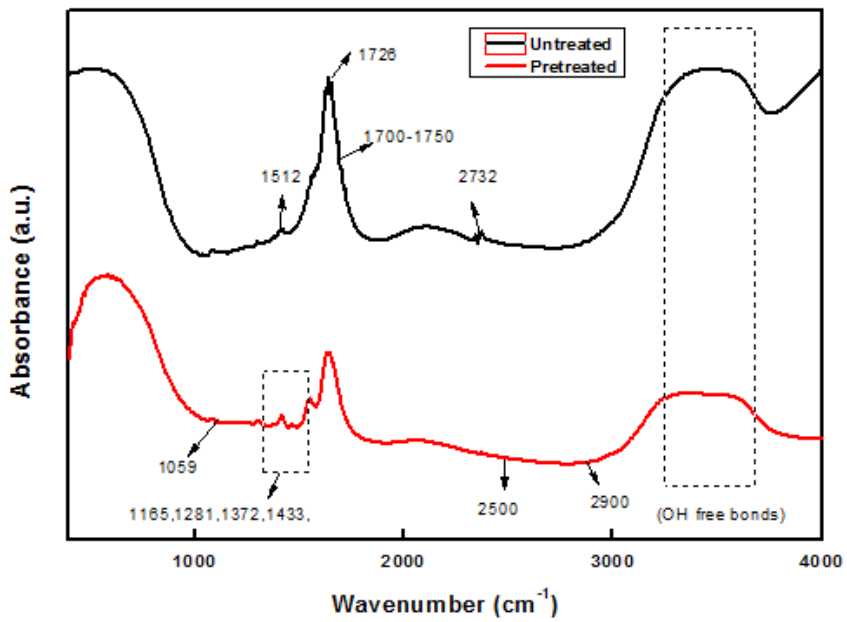


Figure 5.41: FTIR spectra of untreated and pretreated cotton gin waste

Cellulose macrofibrils were shown to be more prominent with an increase in pretreatment time as observed in figure 5.42. XRD analysis reveals the similar crystalline patterns of

pretreated and raw cotton gin waste. The widths at half height of the peaks at 2θ =17º and 26º were similar for all samples except a higher haziness in untreated sample that represents similar crystallite sizes [141]. The respective cellulose crystallinity was measured to be 18% and 26.1%. The crystallinity value is much higher than the individual and mixed fungal pretreatment.

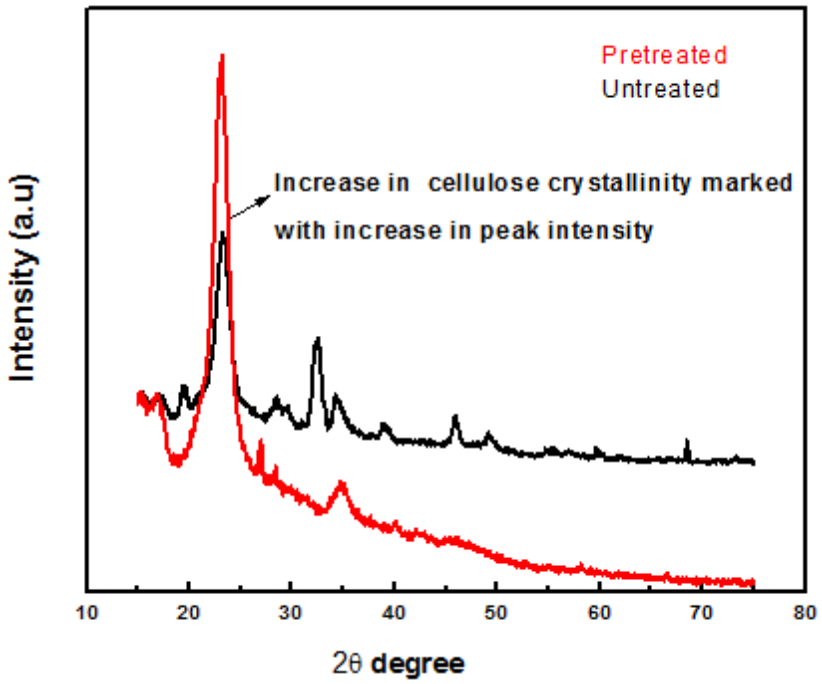


Figure 5.42: XRD analysis of untreated and pretreated cotton gin waste

The morphological characteristic of cellulose and hemicellulose fiber with different severities was assessed by SEM analysis (figure 5.43). The untreated CGW consists of compact fibers distributed over the whole region, where the pretreated biomass shows partially degraded small etched fiber indicating the influence of enzyme hydrolysis. An improved surface area was observed due to the removal of lignin and its associated compounds such as hemicellulose. A significant change in the surface property towards favorable interaction with enzyme has occurred due to pretreatment resulting breakage of the amorphous region of cellulose with retention of crystalline fraction.

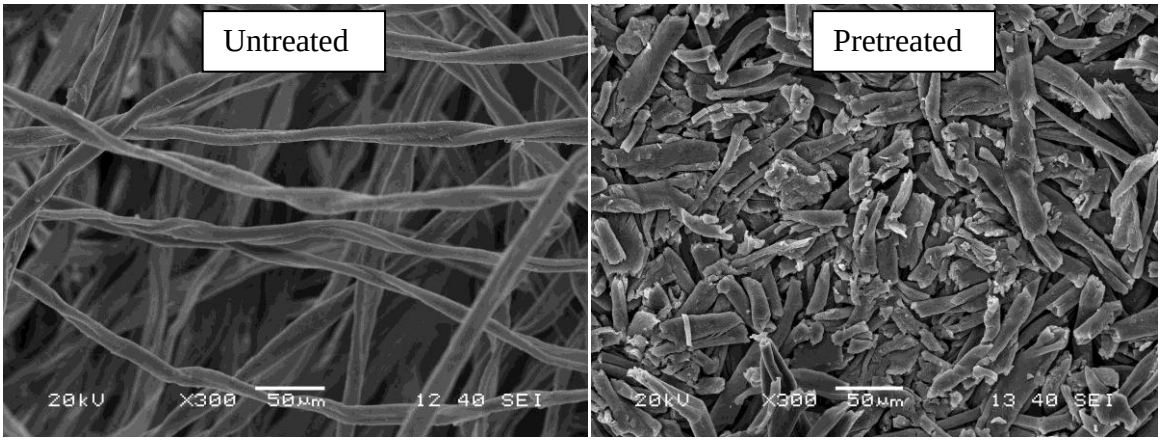


Figure 5.43: SEM of untreated and pretreated cotton gin waste

5.4.7 Enzymatic hydrolysis of delignified cotton gin waste

During the course of enzymatic hydrolysis for 72h, an increase in sugar concentration was observed with time and the rate of hydrolysis became almost constant after 64h. The maximum saccharification yield of 632.72 g/g, 64% saccharification and 34.84 g/l total sugar were obtained due to hydrolysis reaction (Figure 5.44). The individual sugar release was measured as xylose 10.51±0.33 g/l, glucose 23.56±0.67 g/l and arabinose 0.80±0.63 g/l.

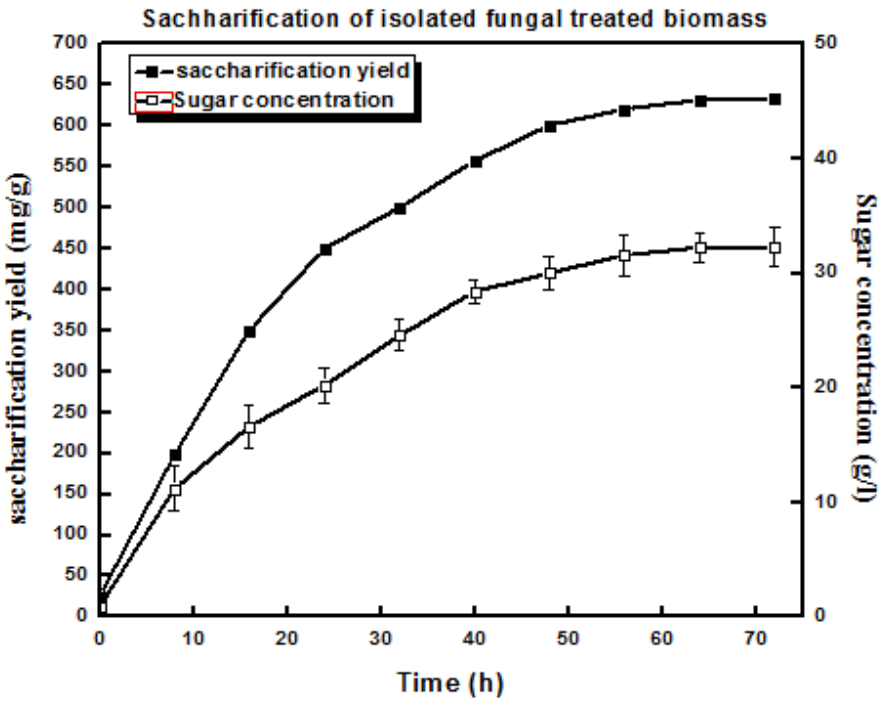


Figure 5.44: Enzymatic saccharification of delignified cotton gin waste by isolated fungal pretreated biomass at 50°C, pH 5 and 150 rpm

5.4.8 Fermentation

The total hydrolysate (34.83 g/l sugars) was fermented by the sequential use of *S. cerevisiae* and *P.stipitis* strains, which resulted in ethanol production of 15.44 g/l, 86% theoretical yield, 0.45 g/g ethanol yield and 1.74 g/l/h productivity after 56h of fermentation in a bioreactor at 200 rpm, 5.5 pH and 30ºC. The reduction in ethanol production observed after 56h may be due to the consumption of accumulated ethanol by the yeast [69]. As previously discussed, ethanol starts accumulation in the medium due to yeast population might have consumed simultaneously sugar and ethanol [145]. A higher biomass yield of 0.35 g/g than mixed culture pretreated waste was obtained after 48h (Table.20).

Table 20: Fermentation of hydrolysate obtained from cotton gin waste by sequential use of *S. cerevisiae* and *P.stipitis* of yeast strains at 30°C, 5.5 pH and 200 rpm

Time (h)	Ethanol conc. (g/l)	Sugar Consumption (g/l)	Ethanol yield (g/g)	Biomass (g/l)	Biomass yield (g/g)
0	0.25	34.85	0.02	0.26	0.01
8	10.83	12.22	0.31	1.62	0.07
16	11.57	10.54	0.33	3.05	0.12
24	12.92	8.37	0.37	4.96	0.19
32	13.81	6.06	0.39	6.21	0.23
40	14.62	4.69	0.41	8.30	0.30
48	15.02	4.07	0.42	9.07	0.35
56	15.44	3.24	0.45	8.99	0.34
64	15.43	3.02	0.45	8.80	0.33
72	15.28	2.89	0.43	8.65	0.31

5.4.9 A comparison study of our experimental results

A comparative study on the experimental results of various conversion processes is shown in Table 21. Among the pretreatment processes, the pretreatment of cotton gin waste with maleic acid was found to be the most efficient pretreatment agent providing the highest pretreatment efficiency and subsequently efficient bioethanol production by fermentation using *S. cerevisiae* and *P. stipitis* yeast strain sequentially followed by the biological pretreatment using *Aspergillus flavus* UNF1. However, the biological pretreatment is

advantageous than the organic acid treatment from an economical point of view by avoiding the step of delignification. The method may also be a promising alternative to the widely used sulfuric acid pretreatment which requires additional delignification and detoxification steps involved in the process. Moreover, the biological method may be a promising alternative to the widely used sulfuric acid pretreatment that requires additional delignification and detoxification steps. The effort may further be given to reduce the higher pretreatment time required for biological pretreatment by genetically modifying the isolated fungal strain thereby making the process more economically viable.

Table 21: A comparative study on the experimental results obtained in various stages of conversion processes of cotton gin waste to bioethanol

Pre-treatment	**Hydrolysis**	**Fermentation**
Composition: Cellulose- 40.3%, Hemicellulose-15% Lignin-19.8%	**Enzymatic hydrolysis**	**Sequential use of yeast strains for un-detoxified maleic acid and detoxified sulfuric acid treated biomass**
Maleic acid : 83% xylose release ⇩ Delignification:88%	67% Saccharification	Ethanol yield-0.48 g/g Productivity- 2.25 g/l/h Biomass yield- 0.30 g/g Ethanol conc. -18.74 g/l
Sulfuric acid: 90% xylose release ⇩ Delignification:89%	66% Saccharification	Ethanol yield-0.45 g/g Productivity-2.11 g/l/h Biomass yield- 0.29 g/g Ethanol conc. -18.9 g/l
Biological pretreatment: *P.cinnabarinus & T. pubscens*:61.2 & 52.14% delignification Cellulose release: 61.9 & 53.5% Hemicellulose release: 70 and 56.8%	56% Saccharification	Ethanol yield-0.42 g/g Productivity-1.06 g/l/h Biomass yield- 0.31 g/g Ethanol conc.- 12.88 g/l
Mixed fungi: ***P.cinnabarinus & T. pubscens*** 63.1% delignification Cellulose release:66% Hemicellulose release:74.5%	59% Saccharification	Ethanol yield-0.43 g/g Productivity- 1.25 g/l/h Biomass yield- 0.31 g/g Ethanol conc. -14.08 g/l
Aspergillus flavus :67.5% delignification Cellulose release:66% Hemicellulose release:74.5%	63% Saccharification efficiency for C6 sugar 86% xylose release(acid +biological)	Ethanol yield-0.45 g/g Productivity- 1.74 g/l/h Biomass yield- 0.35 g/g Ethanol conc. -15.44 g/l

5.5 Comparison of results with published literature

The results obtained in the present research was also compared with the published literature related to the pretreatment, hydrolysis and fermentation study on bioethanol production from cotton gin waste as depicted in Table 22. Venkatramanan et al. 2014 reported 11.8 g/l ethanol concentration by Alkali and acid pretreatment and enzymatic hydrolysis using cellulase extracted from *fusarium* species [165]. In another study, 0.32 g/g bioethanol yield at 72h of fermentation of pretreated cotton gin (ultrasonication + hot water + enzyme) was reported [16]. McIntosh et al. 2014 tested the bioethanol fermentation of cotton gin waste hydrolysates using industrial strain *S. cerevisiae* yielding 85% of theoretical bioethanol yield and 16 g/l bioethanol [143]. A number of hybrid yeast strains by protoplasts fusing from xylose and glucose fermenting yeasts used in the fermentation of CGW hydrolysates resulted in 7.1 ± 0.1 g/l ethanol concentration and 0.44 g /g ethanol yield [5]. Whereas, Fockink et al. 2015 achieved xylose recovery using sulfuric acid at a high temperature in pretreatment followed by enzymatic hydrolysis and fermentation using commercial yeast strains thereby obtained 20g/l ethanol concentration, 0.48 g/g yield and 1.7 g/l/h productivity [144]. Shi et al. 2009 obtained 0.21 g/g ethanol yield using fungal pretreatment of cotton stalk [166].

Overall, as observed from the table, it is hardly possible to make any straight forward comparison among the results due to the variation in several factors in each study, among which the most important are the wide variation in the composition of feedstock and processing conditions. Though, the result reported by Fockink et al. (2015) is apparently the highest performance among all the study, the process has several critical aspects such as the use of undesirable sulfuric acid pretreatment, severity in terms of high temperature and favorable feedstock containing very high cellulosic compound and less lignin content which perhaps the most important one which need to be considered while making comparison [144]. Therefore, it is obvious that a high ethanol production is expected from a raw material with high cellulosic components which is much easier to convert to bioethanol than a feedstock having low cellulosic component and high lignin content which is the most difficult to remove for releasing cellulosic compounds to be available for hydrolysis to release fermentable sugar for bioethanol production. Therefore, we can conclude that the result obtained in our study is more encouraging and interesting which may be beneficial for eventual bioethanol production from cotton gin waste.

Table 22: A comparative study on the experimental results obtained in the present study related to the pretreatment, hydrolysis and fermentation study on bioethanol production from cotton gin waste with the reported literature

Author and year	Pretreatment	Hydrolysis	Fermentation
Venkatramanan et al. 2014 [165]	Cotton waste- Alkali + acid treatment	Cellulase extraction from *fusarium* species	*(S. cerevisiae)* *Ethanol conc. 11.8 g/l*
Shi et al. 2009 [166]	(Cotton stalk) *P. chrysosporium* 40.5% delignification	Cellulase enzymes (commercial)	*(S. cerevisiae)* *EthanolYield:*0.21 g/g
Fockink et al. 2015 [144]	dilute sodium hydroxide(DOE) (CGDglucan76wt%,lignin9. 7%,3% hemicellulose) (CGW- 57% glucan,17% lignin and 7.9% xylose)	Cellulase (commercial) (47.8g/l and 42.5g/l)	*(S. cerevisiae)* *Ethanol conc: 20 g/l* *Productivity:1.7 g/l/h* *EthanolYield:0.48 g/g*
Mcintosh et al. 2014 [148]	CGT- H_2SO_4 treatment (31% glucan, 12.2 xylan) using 180^0C, 12 min, 2% achieved 85% xylose + 23 % cellulose conversion	Cellulase enzymatic (commercial) (40.5 g/l)	*(S. cerevisiae)* *Ethanol conc.: 16 g/l* *Productivity:2.01 g/l/h*
Placido et al.2013 [16]	CGW- Lignolytic enzyme Ultrasonication Liquid hot water	Cellulase and hemicellulase (commercial enzyme)	*(S. cerevisiae)* *Ethanol yield :0.32 g/g* *Productivity:1.46 g/l/h*
Jeoh & Agblevor 2001 [38]	CGW + paper sludge(Steam explosion)	SSF	*(S. cerevisiae)* *Ethanol conc.: 6.71 g/l* *Ethanol yield :0.20 g/l/h*
Kumari & Pramanik 2012 [5]	CGW Dilute H_2SO_4	Enzymatic hydrolysis	*Ethanol conc.*7.1± 0.1 g/l *EthanolYield:*0.44 g/g
Our study	CGW(**maleic acid** pretreatment and ***Aspergillus flavus*** pretreatmnet)	Enzymatic hydrolysis with 41.75 g/l & 34.84 g/l conc. (commercial enzyme)	*S. cerevisiae & P.stipitis* (sequentially) *Ethanol conc.*18.7 g/l & 15.44 g/l *EthanolYield:* 0.48 g/g & 0.45 g/g

Figure 5.45 and 5.46 shows the material balance of all the three conversion processes involving the most effective maleic acid and the isolated strains *Aspergilus flavus* UNF1 pretreatment, hydrolysis and fermentation process.

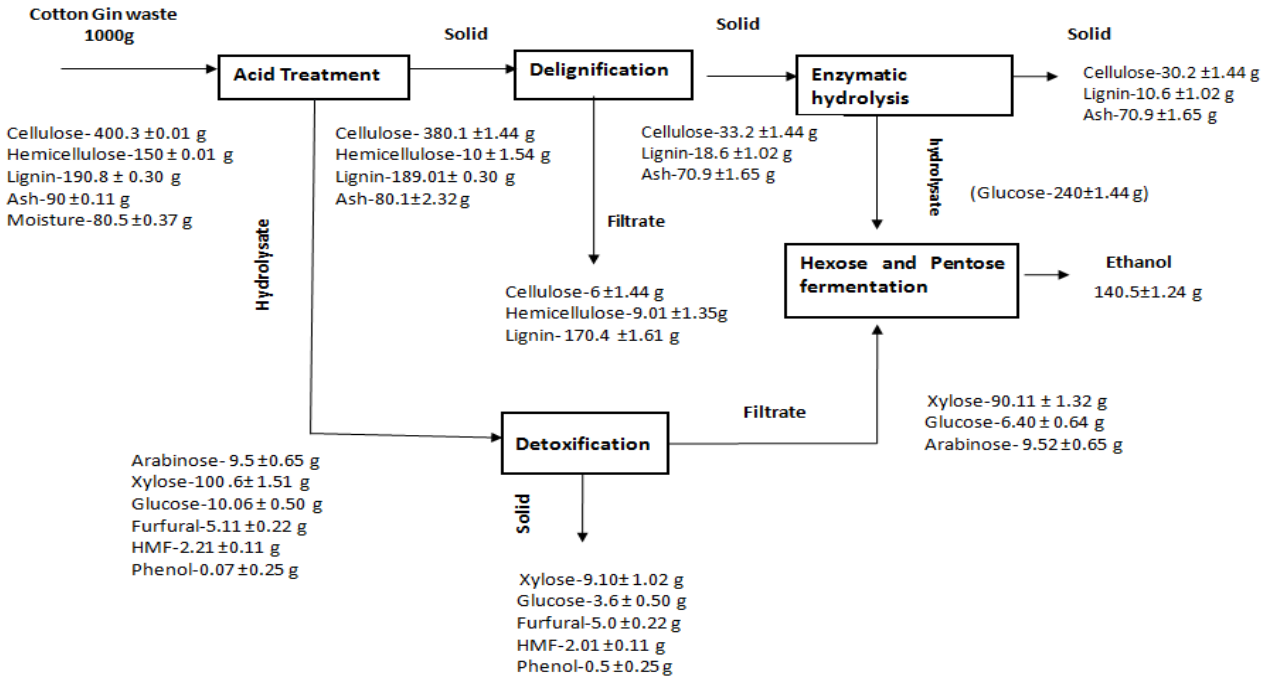


Figure 5.45: Flow chart showing the mass balance of the conversion of cotton gin waste to bioethanol using organic acid pretreatment

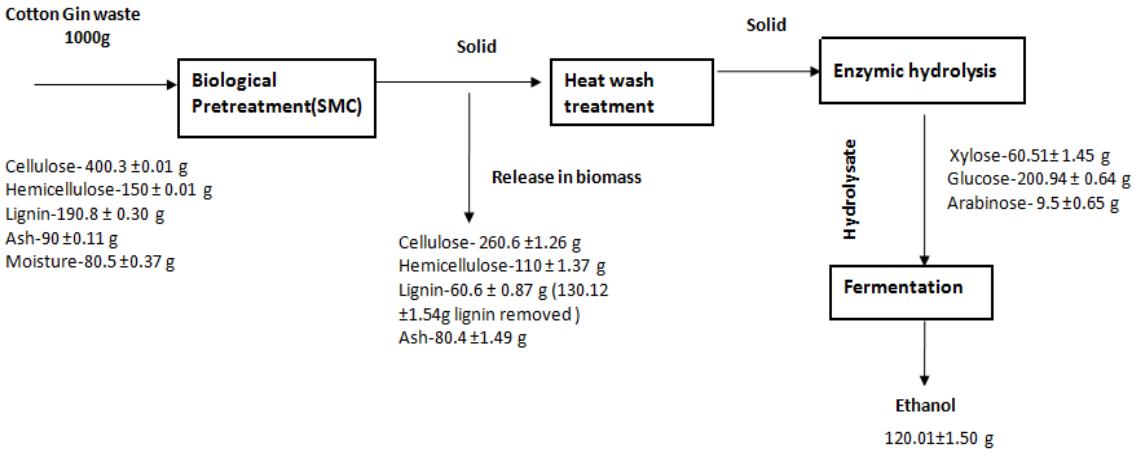


Figure 5.46: Flow chart showing the mass balance of the conversion of cotton gin waste to bioethanol using fungal pretreatment

Chapter 6

Summary and conclusion

6 Summary and Conclusion

In the last decade, a lot of research interest has been generated worldwide for the production of bioethanol from lignocellulosic biomass as an alternative source of transportation fuel. A huge quantity of cotton gin waste, a lignocellulosic biomass, generated in the cotton industry can be a promising raw material for bioethanol production if an effective conversion process is developed. However, a very little work has been done so far for the exploitation of this potential biomass for bioethanol production. There are three major steps involved in the conversion of any lignocellosic waste to bioethanol which includes pretreatment or delignification to release cellulose and hemicelluloses, hydrolysis for converting sugar components to fermentable sugars and fermentation of sugars to bioethanol. While pretreatment of lignocellulosic waste using dilute sulfuric acid is the most efficient and widely used method for lignin removal, besides hazardous, this method suffers from the major drawback of producing toxic by-products which inhibit the growth of microbial strain used for fermentation leading to low ethanol yield. In this context, acid pretreatment using organic acids or biological treatment using fungal strains, seem to be attractive for bioethanol production. Another important challenge lies in the development of an efficient microbial system to convert pentose and hexose sugars efficiently to bioethanol by fermentation.

Keeping th**e** above in view, the main aim of the present research was to investigate the efficiency of the pretreatment of cotton gin waste using organic acid and fungal strains thereby developing an effective pretreatment process for delignification and suitable microbial system for fermentation of sugar components to produce bioethanol from cotton gin waste efficiently. The most important results achieved in this dissertation work are summarized below-

I. The composition analysis has confirmed that cotton gin waste used in this study is a potential feedstock for bioethanol production as it consists of a high percentage of carbohydrates (40.3% cellulose, 15% hemicelluloses, 19.4% lignin).

II. In this phase of work, different batches of pretreatment experiments were performed to evaluate the efficiency of different organic acids namely citric, maleic, oxalic and lactic acid for the release of C5 sugar components from cotton gin waste. Among the four organic acids, maleic acid was found to be the most efficient pretreatment agent providing the maximum release of 83% total C5 sugar with 125.50±0.67 g/g yield at 130°C, 500mM for 45min as optimum pretreatment condition and the major sugar component was xylose (12. 43 g/l, 124.33±1.71 g/g yield, and 82% xylose recovery). The pretreatment efficiency was slightly lower than the most widely used dilute sulfuric acid pretreatment that resulted 88% release of total C5 sugar, 132.08±1.06 g/g sugar yield and 13.11 g/l xylose with 31.13±0.20 g/g yield, and 87% recovery. However, the sulfuric acid pretreatment produced undesirably high level of toxic by-products in comparison to organic acids. The biomasses were delignified efficiently using sodium sulphite in combination with sodium chlorite. The sulfuric acid pretreated biomass was further detoxified to remove inhibitory by-products using overliming and activated charcoal adsorption methods in combination to reduce the inhibitory effect of toxic by-products on yeast growth during fermentation. The delignified maleic acid pretreated and detoxified sulfuric acid pretreated CGW achieved 68 ad 67% scarification in enzymatic hydrolysis. Subsequently, the mixed hydrolysates (C5 & C6) derived from maleic acid pretreated biomass was fermented in a bench top bioreactor. A higher bioethanol production of 18.74 g/l ethanol concentration with 0.48 g/g ethanol yield and 2.25 g/l/h ethanol productivity was observed than the dilute sulfuric acid pretreated biomass by the sequential use of *S. cerevisiae* and *P. stipitis* the most glucose and xylose fermenting yeast strains at optimum fermentation condition of 200 rpm, pH 5.5 and 30°C. The fermentation efficiency was also found to be higher than the use of individual and co-culture of yeast strains.

Therefore, it has been demonstrated that the pretreatment of CGW using maleic acid may be a promising alternative to the widely used dilute sulfuric acid pretreatment. Furthermore, the microbial system using sequential use of *S. cerevisiae* and *P.*

stipitis the most glucose and xylose-fermenting yeast strains is more efficient than the individual and co-culture of yeast strains.

III. In this phase of work, the efficiency of the biological pretreatment of cotton gin waste using four fungal strains such as *Trametes pubscens, Pycnoporus cinnabarinus, Phanerochaete chrysosporium* and *Pleurotus ostreatus* towards the lignin removal was investigated in solid and submerge state of cultivation (SSC and SMC). Among the fungal strains, *Pycnoporus cinnabarinus* has shown higher pretreatment efficiency over other fungi and the maximum 55.5% lignin removal was obtained in SSC. A substantial lignin removal of 52.14% was also observed with *Trametes pubscens*. The pretreatment of CGW using the most efficient *Pycnoporus cinnabarinus* was further optimized by a three-level response surface model (RSM) based on central composite design (CCD), thereby the highest delignification of 61.2 % was achieved in SSC at optimum pretreatment condition of 4.5, 138 rpm and 32°C. Subsequently, the hydrolysis of the pretreated biomass resulted 56% (w/w) saccharification, 563.25 g/g sugar yield and 30.15 g/l total sugar after 64h. The individual sugar component was estimated as 9.57±0.33 g/l xylose, 20.34±0.34 g/l glucose and 0.45±0.11 g/l arabinose. The glucose and xylose rich enzymatic hydrolysates (30.15 g/l) were fermented with sequential use of *S. cerevisiae* and *P. stipitis* to produce bioethanol as the end product. The maximum ethanol production of 12.88 g/l , 0.42 g/g yield, 83% theoretical yield and 1.06 g/l/h productivity were obtained after 56h of fermentation at 30°C, pH 5.5 and 200 rpm.

IV. As it has been reported that co-culture of white rot fungi is more effective for lignin-degradation than the individual culture. However, no work has been reported so far on the use of mixed culture for the pretreatment of CGW. Therefore, the efficiency of *Pycnoporus cinnabarinus* and *Trametes pubscens* on the delignification of cotton gin waste in a mixed culture was investigated with the aim of improving the delignification, thereby increased the release of C5 and C6 sugar components. The maximum delignification of 63.2 % was achieved at optimum pretreatment condition of 4.5, 122 rpm and 35°C. Thus, a higher pretreatment efficiency was shown by mixed fungal pretreated biomass in comparison to individual fungal pretreatment. The hydrolysis of the cellulose and hemicelluloses components provided maximum

59% (w/w) saccharification, 32.25 g/l total sugar and 590.25 g/g saccharification yield after 64h. The corresponding concentration of individual sugar component was calculated as 9.62±0.33 g/l xylose, 22.04±0.34 g/l glucose and 0.65±0.11 g/l arabinose. Subsequently, the maximum bioethanol concentration of 14.08 g/l, 0.43 g/g ethanol yield, 85% of theoretical yield and 1.25 g/l/h productivity was achieved after 56h of fermentation of 32.25 g/l sugar hydrolysate by a sequential use of *S. cerevisiae* and *P.stipitis* yeast strains.

Thus, it has been demonstrated that the pretreatment using mixed culture is more efficient providing higher bioethanol production than the pretreatment using individual strain even *P.cinnabarinus* as the most efficient fungal strain.

V. It was hypothesized that the isolation of lignocellulosic microorganisms that are adapted to the lignocellulosic-rich environment while exhibiting strong lignin degradation ability may be beneficial for biotransformation of lignocellulosic biomass to bioethanol. Therefore, the research work in this phase has focused on the isolation of fungal strains from the soil of a dumping area of cotton ginning mill. Among the isolated fungi, *Aspergillus flavus* (UNF1) was found to be the most efficient fungal strain for the pretreatment of CGW achieving 67.04% lignin removal with the release of 66% and73.5% cellulose and hemicelluloses at 36°C, pH 5 and 110 rpm after 24 days of SMC. Unlike the individual and mixed fungal culture, the pretreatment in SSC was found to be less efficient in comparison to SMC. 34.84 g/l total sugar with 63% saccharification and individual sugar as xylose 10.51±0.33, glucose 23.56±0.67 and arabinose 0.80±0.63 g/l were obtained by the hydrolysis of the pretreated biomass. The total hydrolysate (34.83 g/l sugars) was fermented by *S. cerevisiae* and *P.stipitis* strains sequentially, which resulted an ethanol production of 15.44 g/l, 86% theoretical yield, 0.45 g/g ethanol yield and 1.74 g/l/h productivity after 56h of fermentation. The bioethanol production was higher than that achieved using mixed culture.

Further, though the bioethanol production using *Aspergillus flavus* UNF1 pretreated biomass was slightly lower than the maleic acid pretreated CGW, the biological method is preferable than the maleic acid pretreatment from an economical point of view by avoiding the step of delignification. Similarly, the method may be a

promising alternative to the widely used sulfuric acid pretreatment by avoiding both delignification and detoxification steps involved in the process.

Overall, it has been demonstrated that the pretreatment of cotton gin waste with maleic acid followed by delignification is comparatively more effective providing the maximum pretreatment efficiency with less time and finally bioethanol production than the fungal pretreatment method. A substantial bioethanol production was achieved by biological pretreatment using the *Aspergilus flavus* (UNF1) fungal strain isolated from the soil of the dumping area of cotton gin waste in the cotton industry as a new source. However, the biological pretreatment is favorable than the organic acid treatment from an economical point of view by avoiding the step of delignification. Furthermore, the method may be a promising alternative to the widely used sulfuric acid pretreatment which requires additional delignification and detoxification steps. Thus, it has been demonstrated that the delignification process using *Aspergilus flavus* UNF1 as pretreatment agent and the microbial system involving the sequential use of *S. cerevisiae* and *P. stipitis* yeast strains for fermentation may pave the way for large-scale bioethanol production from cotton gin waste in future.

Suggested Future Study

i. Efforts may be given to improving bioethanol production by developing genetically modified *Aspergilus flavus* and yeast strains.
ii. Kinetic models may be developed from the experimental results for improved fungal and yeast growth thereby increased bioethanol production.
iii. Details cost analysis needs to be carried out to access the feasibility of different conversion processes by conducting the study at pilot scale for the large scale production of cotton gin waste.

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